The influence of tannins on the extrafloral nectar characteristics and insect mutualists of *Vicia faba* L.

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Abstract

The main objective of this research project was to investigate the influence of tannins on the extrafloral nectar characteristics and insect mutualists of *Vicia faba* L. Tannin-free cultivars of *V. faba* have become increasingly popular in Western Canada due to the greater digestibility of their protein by monogastrics; however, the effect of their lack of tannins on mutualistic insects is unknown. Tannin-rich cultivars of *V. faba* produce characteristic dark spots on the flowers' wing petals, and on the stipular extrafloral nectaries (EFNs), which are often used by insects to help locate nectaries. Tannin-free *V. faba* cultivars lack these nectar guides and spots though, and may be unable to attract as many beneficial insects to the EFNs for herbivore-control purposes, and to the flowers for pollination. Accordingly, this study investigated two tannin-rich (Fatima, SSNS-1) and two tannin-free cultivars (Snowbird, Snowdrop). Extrafloral nectar characteristics were also examined, as the production of tannins can be metabolically expensive, and could come at the cost of extrafloral nectar secretion. Tannin-free cultivars are therefore expected to attract fewer beneficial insects due to their unmarked flowers and EFNs, unless the lack of tannins corresponds with an increased production of nectar or nectar sugars.

To examine the effects of tannins on the insect mutualists of *V. faba*, surveys of insect visitors to the EFNs and flowers were conducted throughout the summers of 2013 and 2014. The vast majority of insect visitors to EFNs were ants (Formicidae), followed by ladybird beetles (Coccinellidae), flies of *Camptoprosopella borealis* Shewell (Lauxaniidae), and predatory (Vespidae) and parasitoid (Ichneumonidae) wasps, whereas the bees *Apis mellifera* L. and *Bombus nevadensis* Cresson were the most common visitors to the flowers. The cultivars which those species were present or absent at during surveys were analyzed using generalized mixed models. The results did not support any consistent differences in insect visitors to plants with tannins, compared to those without, suggesting that the marked difference in the visibility of EFNs on tannin-rich cultivars is not essential for EFN recognition by many insect species. Furthermore, insect visits to EFNs occurred at a highly conserved relative location along the stem, due to a probable increase in nectar production a short distance from the shoot apex. For future reference, stipules at this node were termed the Primary Active EFNs.

Extrafloral nectar characteristics were studied in a growth chamber through a combination of nectar sampling by microcapillaries and refractometer measurements to examine nectar volume and sugar concentration, respectively, as well as high performance liquid

chromatography to measure the proportion of each of the nectar sugars present. On average, the extrafloral nectar per stipule ± S.E.M had a volume of 0.363 ± 0.021 µL, a nectar sugar concentration (by weight) of 32.5 ± 1.3 %, a nectar sugar mass of 137.6 ± 10.0 µg, and a sugar composition of 54.4 ± 1.0% glucose, 31.1 ± 1.0% fructose, and 14.5 ± 1.0% sucrose. Although extrafloral nectar characteristics varied between cultivars, the variability did not appear dependent on tannin presence or absence, nor did it appear to influence the presence or absence of the abundant ant species *Formica neoclara* Emery, *F. podzolica* Francoeur, and *Lasius pallitarsis* (Provancher), at different cultivars in the field. The increased digestibility provided by tannin-free cultivars of *V. faba* to monogastrics such as chickens, therefore, does not appear to come at the cost of reduced visitation to the plants by ants and other beneficial insects.

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1.1 *Vicia faba* L.

Vicia faba, commonly known as the faba, fava, field, or broad bean, is a crop plant grown across the world, and belongs to the family Fabaceae (previously known as Leguminosae). As one of many legume crops grown in Saskatchewan, Canada, *V. faba* has been commercially produced in Western Canada since 1972 (McVicar *et al.* 2008). The seeds of faba beans are often sold for either human consumption or as animal feed. Like many other pulse crops, *V. faba* is grown in crop rotations to provide the soil with additional nitrogen, and can be used as a silage crop as well (McVicar *et al.* 2008). Plants of *V. faba* have thick stems, can grow axillary tillers, and have compound leaves arising from each node of the plant. A pair of stipules also grows from the stem at the base of each leaf (Fig. 1.1a,b). Each stipule has an extrafloral nectary (EFN) that produces nectar attractive to a variety of insects, including those that parasitize or prey on herbivorous insects. Additionally, pollinating insects are attracted to the plant by floral nectar, which is produced by flowers in a raceme inflorescence (Fig. 1.1c,d).

1.1.1 Varieties and cultivars of *V. faba*

The species *V. faba* is composed of 3 main varieties: *V. faba* var. *major* Harz (Chinese broad bean), *V. faba* var. *minor* Harz (field/faba bean), and *V. faba* var. *equina* Steudel (horse bean) (Aouar-sadli *et al.* 2008, Bond and Kirby 1999, Kendall and Smith 1975, McVicar *et al.* 2008, Pierre *et al.* 1996). All cultivars used in this study are *V. faba* var. *minor*, which is the only variety for which Canada has significant exports (McVicar *et al.* 2008). Currently, 13 cultivars of *V. faba* are registered for cultivation in Canada (CFIA 2016), of which Snowbird was the most commonly grown cultivar in Saskatchewan in 2015. Several of the cultivars chosen for this project were developed locally by the University of Saskatchewan's Crop Development Centre (CDC), including CDC Fatima, CDC Snowdrop, and CDC SSNS-1. Fatima is the industry standard for export trade of food-type faba beans, and has large seeds with normal seed coats (SPG and CDC 2013). Both Fatima and SSNS-1 cultivars produce tannins, whereas Snowdrop is the first small-seeded zero tannin cultivar, and was released in 2012 by the Saskatchewan Pulse Grower's Variety Release Program. Snowbird, another tannin-free cultivar used in this study, was developed by Innoseeds B.V. of Vlijmen, Netherlands.

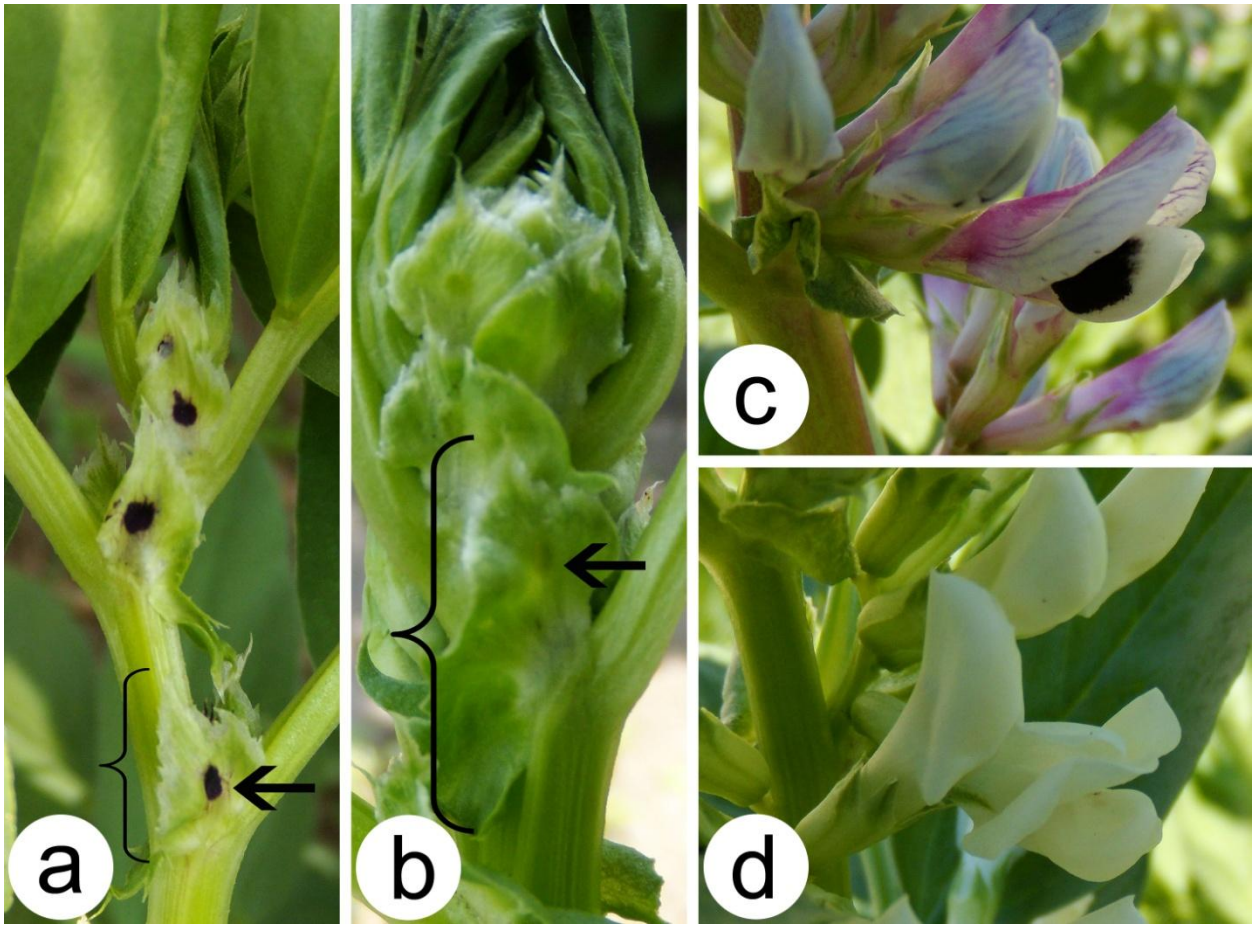


Figure 1.1 Stipular extrafloral nectaries (EFNs) near the apex of the stem (a,b) and flowers (c,d) of *Vicia faba*. Brackets indicate the location of stipules, and arrows point to the location of EFNs, specifically the Primary Active EFN for (a). Represented are plants that contain tannins (a,c) and lack tannins (b,d).

Cultivars of *V. faba* containing tannins have several associated phenological traits, including dark coloured EFNs (Fig. 1.1a), colouration on the flowers (Fig. 1.1c), and coloured seed coats. In contrast, those cultivars which are tannin-free display colourless EFNs (Fig. 1.1b), as well as both white flowers (Fig. 1.1d) and seeds (Marquardt *et al.* 1978). The tannin-free cultivars are popular among crop growers due to the increased nutritive value of the seeds as animal feed, because the seeds have both more digestible protein and a higher energy value for animals such as chickens (Brufau *et al.* 1998, Crépon *et al.* 2010, Vilariño *et al.* 2009).

Several different varieties and cultivars have been investigated in past studies of *V. faba*'s floral visitors, including *V. faba* var. *equina*, grown by Pierre *et al*. (1996) and Tasei (1976). *Vicia faba* var. *major* was also used by some authors, with cultivars Aquadulce, Reina Blanca and experimental lines grown by Bond and Kirby (1999), and unspecified cultivars by Aouar-

sadli *et al.* (2008) as well as Benachour *et al.* (2007). Additionally,the cultivar Maris Bead of *V. faba* var. *minor* was used by Kendall and Smith (1975), whereas cultivars Ackerperle, Diana, Erfordia, and Herz Freya of *V. faba* var. *minor* were grown by Malaipan (1979), with Ackerperle also having previously been used by Poulsen (1973). Additionally, Malaipan (1979) and Nuessly *et al.* (2004) also studied EFNs in the above mentioned cultivars. The EFN visitors of cultivar Ipro of *V. faba* var. *major* were examined by Bugg *et al.* (1989), however, the majority of other studies reporting EFN visitors did not specify the cultivar of *V. faba* grown.

1.1.2 Reproductive characteristics of *V. faba*

Despite *V. faba*'s flowers being both hermaphroditic and self-compatible, it is known that cross-pollination is advantageous for *V. faba* (Crofton 1996, Mussalam *et al.* 2004). The at least partially allogamous flowers rely on bees for pollination, particularly bees of the family Apidae, including *Anthophora*, *Apis*, *Bombus*, and *Eucera* (Benachour *et al.* 2007, Bond and Kirby 1999). Many other species of insects are also known to visit the flowers, although are likely not all effective pollinators (Table 1.1). Flowering of plants typically occurs 45-60 days after planting and continues for up to 58 days (McVicar *et al.* 2008), although individual flowers only bloom for three to five days (Kołtowski 1996a). Each axillary raceme generally bears between two and six flowers, the petals of which typically have a white background colour (Knott 1990). Cultivars with tannins sometimes have additional pink to purple anthocyanin pigmentation present on sepals and standard petals, and often have a large black or brown spot on the wing petals as well. *Vicia faba* is diploid with 2n = 2x = 12, whereas most related species, such as *V. narbonensis* L., are 2n = 2x = 14, and all interspecific hybrids using *V. faba* have failed (Ladizinsky 1975).

1.2 Nectaries

The stipular nectaries of *V. faba* are located as often contrasting dark purple or brown circles on the green stipule's abaxial surface, and are composed of hundreds of both secretory and non-secretory trichomes (Davis *et al.* 1988, Heneidak and Hassan 2007). Secreted extrafloral nectar forms exposed drops over the stipular nectaries. Floral nectaries can also be found on the plant, and each appears as a pale, zygomorphic green disk surrounding the base of the gynoecium. Phloem alone supplies this floral, multicellular outgrowth (Davis *et al.* 1988). The nectar then gathers around the gynoecium, at the bottom of the androecium`s cavity (Davis and

Table 1.1 Insect visitors to the flowers of *Vicia faba* recorded from previous studies.

Order	Family	Genus or Species and Authority	Reference
Coleoptera	Cantharidae	*Chauliognathus marginatus* (Fabricius)	Nuessly *et al.* (2004)
	Nitidulidae	*Carpophilus* sp.	Malaipan (1979)
	Scarabaeidae	*Anomala marginata* (Fabricius)	Nuessly *et al.* (2004)
		Euphoria sepulcralis (Fabricius)	
		Trigonopeltastes delta Forster	
Hemiptera	Anthocoridae	*Orius tristicolor* (White)	Malaipan (1979)
Hymenoptera	Andrenidae	*Andrena ovatula* (Kirby)	El-Berry *et al.* (1974)
	Apidae	*Anthophora acervorum* (Linnaeus)	Tasei (1976)
		A. dispar Lepeletier	Aouar-sadli *et al.* (2008)
		A. plumipes Pallas	Bond & Kirby (1999)
		Apis mellifera Linnaeus	Aouar-sadli *et al.* (2008), Benachour *et al.* (2007), Bond & Kirby (1999), Kendall & Smith (1975), Malaipan (1979), Pierre *et al*. (1996)
		Bombus borealis Kirby	Malaipan (1979)
		B. distinguendus Morawitz	Poulsen (1973)
		B. griseocollis (DeGeer)	Malaipan (1979)
		B. hortorum Linnaeus	Bond & Kirby (1999), Kendall & Smith (1975), Poulsen (1973)
		B. lapidarius (Linnaeus)	Poulsen (1973), Tasei (1976)
		B. lucorum Linnaeus	Kendall & Smith (1975)
		B. pratorum (Linnaeus)	Tasei (1976)
		B. pascuorum Scopoli	Bond & Kirby (1999), Kendall & Smith (1975), Poulsen (1973), Tasei (1976)
		B. ruderatus (Fabricius)	Benachour *et al.* (2007)
		B. rufocinctus Cresson	Malaipan (1979)
		B. terrestris Linnaeus	Aouar-sadli *et al.* (2008), Benachour *et al.* (2007), Kendall & Smith (1975), Pierre *et al*. (1996), Poulsen (1973)
		B. terricola Kirby	Malaipan (1979)
		B. vagans Smith	
		Eucera alternans Brullé	Benachour *et al.* (2007)
		E. numida Lepeletier	Aouar-sadli *et al.* (2008), Benachour *et al.* (2007)
		E. pulveracea Dours	Aouar-sadli *et al.* (2008)

Table 1.1 (cont.)

Order	Family	Genus or Species and Authority	Reference
		Eucera tuberculata (Fabricius)	Tasei (1976)
		Tetralonia lanuginosa Klug	El-Berry *et al.* (1974)
		Thyreus sp.	
		Xylocopa aestuans (Linnaeus)	
		X. micans Lepeletier	Nuessly *et al.* (2004)
		X. valga Gerstaecker	Aouar-sadli *et al.* (2008)
		X. violacea Linnaeus	Aouar-sadli *et al.* (2008), Benachour *et al.* (2007)
	Chrysididae	*Chrysis* sp.	Nuessly *et al.* (2004)
	Halictidae	*Agapostemon splendens* Lepeletier	
		Halictus sp.	
		Lasioglossum clavipes Dours	Aouar-sadli *et al.* (2008)
		L. villosullum Kirby	
	Megachilidae	*Megachile inermis* Provancher	Malaipan (1979)
		M. sicula (Rossi)	El-Berry *et al.* (1974)
		Osmia hebraea Benoist	Benachour *et al.* (2007)
	Sphecidae	*Liris* sp.	Nuessly *et al.* (2004)
	Vespidae	*Eumenes fraternus* Say	
		Pachyodynerus nasidens (Latreille)	
		Polistes dorsalis (Fabricius)	
		P. major Beauvois	
		P. metricus Say	
Lepidoptera	Hesperiidae	*Lerema accius* (J. E. Smith)	
	Nymphalidae	*Danaus plexippus* (Linnaeus)	Malaipan (1979)
		Vanessa atalanta Linnaeus	
	Pieridae	*Colias* spp.	
		Pieris rapae Linnaeus	
Thysanoptera	Thripidae	*Frankliniella bispinosa* (Morgan)	Nuessly *et al.* (2004)
		F. insularis (Franklin)	
		F. kelliae (Sakimura)	
		F. tritici (Fitch)	Malaipan (1979)

Gunning 1992). Floral nectar flows through non-regulatory, modified stomata, which are highly concentrated on the nectary's surface (Davis and Gunning 1993).

1.2.1 Distribution of extrafloral nectaries in related taxa

Vicia faba is one of the 1069 species of Fabaceae which has EFNs; this family represents over a quarter of the known species with EFNs, which is more than any of the other approximately 100 families with EFNs (Koptur 1992, Weber and Keeler 2013). Altogether the Fabaceae family has 112 different genera with EFNs, with the genera *Inga* and *Acacia* (*sensu lato*) including nearly half of that family's species with EFNs (Keeler 2008). EFNs in Fabaceae

can be found in a wide variety of locations, including the leaves, petioles, stipules, stems, pedicels and other sites nearer to the flower.

Of the 140 species belonging to the genus *Vicia* L. (Heneidak and Hassan 2007), three species are native to Canada, and around nine have been introduced (Small 2013). Of these Canadian species, EFNs are known from *V. faba* (Davis *et al.* 1988), *V. sativa* L. (Katayama and Suzuki 2011, Koptur 1979), *V. sepium* L. (Lenoir and Pihlgren 2006), and *V. villosa* Roth (Wrona 1971). The EFNs of *V. sativa* and *V. sepium* are found on the abaxial surface of the stipules (Stpiczynska 2000), just as they are on *V. faba*. Other species of *Vicia* found in Canada lack EFNs, including *V. americana* Muhl. ex Willd., *V. caroliniana* Walter, *V. cracca* L., *V. hirsuta* (L.) Gray, *V. lathyroides* L., *V. nigricans* Hook. and Arn. (USDA 2013), *V. pannonica* Crantz (Small 2013) and *V. tetrasperma* (L.) Schreb. (USDA 2013). Of the *Vicia* species found in Canada, *V. americana, V. cracca* (USDA 2013), and *V. faba* (McVicar *et al.* 2008) are present in Saskatchewan, though only *V. americana* is native to the province (Harms 2006). *Vicia faba* has no known wild relative, and even the most closely related *Vicia* species are much more related to one another than to *V. faba* (Emshwiller *et al.* 2011). Without a known wild relative, the origin of *V. faba*'s domestication is uncertain; however, the strongest evidence to date has indicated its domestication started in the Near East, at least 9300 BP (Hancock 2012).

1.2.2 Insect visits to EFNs

In some plants, herbivore attacks can induce additional nectar production by EFNs (Heil 2004), and in others, including certain cultivars of *V. faba*, an increase in the production of EFNs occurs when the plant is damaged (Mondor and Addicott 2003, Mondor *et al.* 2013). In contrast to herbivory, nectar consumption by insects does not harm the plant, and the possession of EFNs appears to be an adaptation to attract predatory and parasitoid insects (Koptur 1992). The EFNs of *V. faba* are known to attract a range of beneficial insects, including predators such as ants and beetles, as well as a variety of parasitoid wasps (Table 1.2). The attracted wasps and ants are known to reduce herbivory through the elimination of some of the herbivorous insects in the area (Cuautle and Rico-Gray 2003, Dequech *et al.* 2010, Kost and Heil 2005, Pemberton and Lee 1996). For *Catalpa bignonioides* Walter, the entire sequence of events involved in herbivore defence has been investigated; within 36 hours of herbivory, fewer caterpillars remained on plants with increased ant densities, whose numbers had swollen during a two- to three-fold

Table 1.2 Insect visitors to the extrafloral nectaries of *Vicia faba* recorded from previous studies.

Order	Family	Genus or Species and Authority	Reference
Coleoptera	Cantharidae	*Cantharis fulvicollis* Fabricius	Hetschko (1908)
	Coccinellidae	*Adalia bipunctata* Linnaeus	
		Coccinella septempunctata Linnaeus	
		C. transversoguttata Mulsant	Malaipan (1979)
		Coccinula quatordecimpustalata Linnaeus	Hetschko (1908)
		Hippodamia convergens Guérin-Méneville	Malaipan (1979)
		H. tredecimpunctata (Linnaeus)	
Diptera	Anthomyiidae	-	Bugg *et al.* (1989)
	Calliphoridae	*Pollenia vespillo* Fabricius	Hetschko (1908)
	Muscidae	*Musca corvina* Linnaeus	
	Sarcophagidae	*Sarcophaga carnaria* Linnaeus	
	Syrphidae	*Syrphus* spp.	Malaipan (1979)
	Tachinidae	-	Bugg *et al.* (1989)
Hemiptera	Miridae	*Lygus pratensis* Fabricius	Hetschko (1908)
Hymenoptera	Apidae	*Apis mellifera* Linnaeus	Free (1962) & Hetschko (1908)
	Chalcididae	*Brachymeria* sp.	Nuessly *et al.* (2004)
		Conura sp.	
	Evaniidae	-	Bugg *et al.* (1989)
	Formicidae	*Lasius niger* Linnaeus	Hetschko (1908) & Katayama & Suzuki (2003)
		Myrmica laevinodis Nylander	Hetschko (1908)
		Tetramorium caespitum Linnaeus	
		T. tsushimae Emery	Katayama & Suzuki (2004)
	Ichneumonidae	*Banchus inermis* (Provancher)	Bugg *et al.* (1989)
		Cratichneumon vescus (Provancher)	
		Ctenichneumon minor Heinrich	
		Ectopimorpha wilsoni (Cresson)	
		Gambrus ultimus (Cresson)	
		Ichneumon laetus (Brulle)	Bugg *et al.* (1989) & Malaipan (1979)
		Neotypus nobilitator nobilitator (Gray)	Bugg *et al.* (1989)
		Pimpla aequalis (Cresson)	
		Platybus clarus (Cresson)	
		Polytribax contiguus (Say)	
		Pterocormus ambulatorius (Fabricius)	
		P. annulatorius (Fabricius)	
		P. nigrovariegatus (Provancher)	
		P. subdolus (Cresson)	

Table 1.2 (cont.)

Order	Family	Genus or Species and Authority	Reference
		Rubicundiella perturbatrix Heinrich	
		Setanta compta (Say)	
		Stenobarichneumon duplicans Heinrich	
		Vulgichneumon brevicinctor (Say)	
		V. mimicus (Cresson)	
		V. terminalis (Cresson)	
	Vespidae	*Dolichovespula arenaria* (Fabricius)	Malaipan (1979)
		D. norvegicoides (Sladen)	
		Polistes fuscatus pallipes Lepeletier	Bugg *et al.* (1989)
		Vespula alascensis (Packard)	Malaipan (1979)
		V. consobrina (Linnaeus)	
		V. maculifrons (Buy)	
Neuroptera	Chrysopidae	*Chrysopa* sp.	

increase in extrafloral nectar sugar production (Ness 2002). In addition to the herbivore deaths resulting from predation and parasitism, wasps and ants can also decrease herbivore numbers on plants through non-consumptive effects. Patrolling and hunting activities around the host plant can cause aphids to drop off the plant (Fill *et al.* 2012), and delay the foraging time available to many insects, including larval Lepidoptera (Cuautle and Rico-Gray 2003). Although floral nectar is also produced by many plants with EFNs, the insects attracted to extrafloral nectar are almost entirely different than those insects which visit flowers of the same plant, due to differences in nectary accessibility, as well as nectar composition.

1.2.2.1 Accessibility of EFNs

In general, EFNs are much more exposed than floral nectaries, and therefore allow easier access to nectar for a wide variety of insects which have shorter mouthparts unsuited for extracting nectar from a tubular flower (Hespenheide 1985). Most predatory and parasitoid Hymenoptera are equipped with relatively unspecialized mouthparts, and are common visitors to the EFNs of many plants including *Byttneria aculeata* Jacquin (Hespenheide 1985), *Gossypium hirsutum* L. (Stapel *et al.* 1997), *Phaseolus lunatus* L. (Kost and Heil 2005), *Prunus persica* L. Batsch (Mathews *et al.* 2011), *Solanum volubile* Sw. (Gentry 2003), and *Turnera ulmifolia* L. (Cuautle and Rico-Gray 2003). Predatory and parasitoid insects are also known to feed from flowers with relatively exposed nectaries, such as many umbelliferous flowers (Bugg and Wilson 1989, Çoruh and Çoruh 2008, Jervis *et al.* 1993, Leius 1960, Maingay *et al.* 1991). These easily accessed droplets of nectar act as important sources of food for many insects which otherwise

would be considered entirely predatory or parasitoid (Bugg *et al.* 1987, Lewis *et al.* 1998, Pemberton and Vandenberg 1993, Röse et al. 2006).

1.2.2.2 Nectar preferences of predatory and parasitoid insects

In addition to being more accessible, extrafloral nectar composition can also vary significantly from that of floral nectar. Amino acid complements, as well as sugars, can vary significantly between floral and extrafloral nectar of the same plant (Baker *et al.* 1978, Davis *et al.* 1988). The dissacharide sucrose is found as a primary component in *V. faba*'s floral nectar but only in trace amounts in the extrafloral nectar (Davis *et al.* 1988). Sucrose is not as easily located by some parasitoid wasps through chemosensory means as other food sources, such as extrafloral nectar or honey (Röse *et al*. 2006). This discrepancy may be due to sucrose's lower value as a food source to parasitoid wasps, because although sucrose is known to increase wasp longevity (Wäckers 2001), it does not necessarily allow wasps to fly any farther. The consumption of sucrose solutions by *Cotesia glomerata* L. does not allow the wasps to significantly increase the total distance they can fly any more than water does (Wanner *et al.* 2006). However, the female wasps were able to extend their total flight distances using a variety of other food sources, including extrafloral (*V. faba*) and floral (*Anethum graveolens* L. and *Origanum vulgare* L.) nectar, as well as honey.

The lack of benefits received by parasitoid wasps drinking sucrose rich solutions may be due to an absence of certain sugar digesting enzymes, such as invertase. Invertase, an enzyme used to cleave sucrose into its constituent sugars glucose and fructose, is normally not produced by the ant *Pseudomyrmex ferrugineus* (F. Smith) which has developed close, mutualistic associations with extrafloral nectar producing *Acacia* plants (Kautz *et al.* 2008). Several other species of ants also have very low invertase activity (Ayre 1967), so these ants must therefore rely on the invertase activity of the plant to convert sucrose into digestible monosaccharides (Heil *et al.* 2005a). In the case of *Acacia*, the extrafloral nectar itself contains inhibitory enzymes, which disable invertase activity in the specialist *Pseudomyrmex*, and prevents those ants from exploiting other plants' sucrose rich nectar (Heil *et al.* 2014). However, in many species of ants, nectar rich in sucrose is often preferred, although some ants do not show any preference for specific nectar sugars (Blüthgen and Fiedler 2004, Cornelius *et al.* 1996). Extrafloral nectar can also be rich in amino acids (Shenoy *et al.* 2012), which can influence the

nectar preferences of ants as well (Blüthgen and Fiedler 2004). Although nectar composition is generally thought to be controlled by the plant secreting the nectar, ants have also been known to introduce yeasts into floral nectar, which can hydrolyze sucrose into monosaccharides (De Vega and Herrera 2013). Additionally, the introduced microbes can result in an increase in fructose relative to glucose, possibly due to the more rapid metabolism of glucose by some fungi.

1.2.2.3 Visibility of extrafloral nectaries

For the parasitoid and predatory insects to drink extrafloral nectar, they must first find the EFNs. Nectary detection can in part be accomplished through the insects' chemosensory abilities, but many EFNs, including those of *V. faba* cultivars that produce tannins, are also coloured darkly, which provides a visual cue for insects attempting to locate the EFNs (Koptur 1992). Most insects have colour vision, including ants (Aksoy and Çamlitepe 2014, Çamlitepe and Aksoy 2010), and even others such as certain parasitoid wasps can at least perceive shades of gray due to the presence of a single spectral receptor type in their eyes (Bradbury and Vehrencamp 1998, Briscoe and Chittka 2001). Some of these parasitoids rely on visually distinguishing contrasted areas to determine the location of hosts for oviposition (Brown *et al.* 1998, Fischer 2002, Wäckers and Lewis 1999), although vibrational cues are also a factor in some cases (Fischer *et al.* 2001, Kroder *et al.* 2007). Ants are also known to use visual cues extensively during foraging (Cammaerts 2012, Evison *et al.* 2008, Schultheiss *et al.* 2015). The lack of contrasting pigment on the EFNs of tannin-free *V. faba* cultivars could result in fewer of these predatory and parasitoid insects finding the EFNs, compared to regular tannin-containing cultivars.

1.3 Objectives

This project sought to increase our knowledge of the effect of tannins on *Vicia faba*'s extrafloral nectar characteristics and insect mutualists through three main objectives:

1) to examine whether the presence or absence of tannins in *V. faba* is correlated with potential differences in the composition and frequency of insects attracted to the extrafloral and floral nectaries of several *V. faba* cultivars;

2) to investigate the differences in extrafloral nectar composition, volume, and concentration between *V. faba* cultivars with and without tannins; and

3) to explore how extrafloral nectary pigmentation may affect the frequency and composition of insects visiting nodes of different relative positions along the stems of *V. faba*, throughout its growing season.

2 Insect visits to floral and extrafloral nectaries of *Vicia faba*

2.1 Introduction

Although both the flowers and stipules of *V. faba* produce nectar, the insects attracted to the nectar are distinctly different depending on the type of nectary. Extrafloral nectaries on *V. faba* are exposed and easily accessed by predatory and parasitoid insects, whereas the floral nectaries are cryptic and accessible only to those insects with long tongues. Therefore, the floral morphology of *V. faba* results in predatory and parasitoid insects being unable to access floral nectar, except by possibly chewing a hole through the base of the flower. Bees, however, have been known to drink the extrafloral nectar of plants, including *V. sativa* (Koptur 1992), although they may not have been effective pollinators of those particular plant species. The probability of insects visiting both extrafloral and floral nectaries of the same plant species is further reduced by the differences in nectar composition typically found between nectary types (Baker *et al.* 1978, Elias *et al.* 1975, Keeler 1977). Neither the occurrence of predatory and parasitoid insects at floral nectaries, nor the use of EFNs by pollinators other than *Apis mellifera* (Free 1962, Hetschko 1908), appear to be reported for *V. faba* in literature, so overlap between species visiting extrafloral and floral nectaries is probably uncommon. Besides *Apis*, the only other genus with a species occurring at both floral nectaries and EFNs was the predatory wasp genus *Polistes*, but the nectary visits involved different species (Table 1.1, 1.2). Insects drinking at either extrafloral or floral nectaries will therefore be treated separately, as the two nectary types predominantly serve to attract insects with different roles in relation to the plant (Koptur 1992).

2.1.1 Insects found consuming the extrafloral nectar of *V. faba*

Although no other studies were found to compare the insects associated with tannin and tannin-free cultivars of *V. faba*, a few studies have examined the insects associated with the darkly-pigmented EFNs of regular cultivars. Adult parasitoid wasps are one commonly encountered group at EFNs (Table 1.2), and on *V. faba* in southern Florida, eight different parasitoid species were found to be associated with the plant (Nuessly *et al.* 2004); however, only two of these, both small chalcid wasps (family Chalcididae), were observed at the EFNs. In contrast, a study from Massachusetts detected almost 60 individuals of Ichneumonidae, composed of at least 20 different species, feeding at the EFNs of the *V. faba* cultivar Ipro (Bugg *et al.* 1989). There was no sex bias apparent for the wasps in that study, as 28 female and 30

male ichneumonid wasps were recorded. In addition, several families of flies (Diptera), as well as some predatory wasps, were found at the EFNs. Additionally, several species of the predatory wasps *Vespula* and *Dolichovespula* were found consuming extrafloral nectar in Manitoba, Canada (Malaipan 1979). The former study, as well as both of the studies from the United States of America, relied on qualitative sampling techniques when examining visitors to the EFNs, so EFN visitation rates have not been previously calculated.

Other predatory insects have also been found at the EFNs of *V. faba* (Table 1.2), including Coccinellidae (ladybird beetles) and Formicidae (ants). These groups of insects tend to be observed at the EFNs of many plants, such as the 41 coccinellid species that have been found feeding at the EFNs of 15 plant families (Pemberton and Vandenberg 1993). Several species of coccinellid beetles have been located on *V. faba* (Hetschko 1908), and additional coccinellids (*Coccinella quinquepunctata* L. and *Hippodamia convergens* Guerin) have also been found feeding at the EFNs of the other *Vicia* species (Pemberton and Vandenberg 1993). *Vicia faba* has also been used as a model system in experiments involving feeding extrafloral nectar to coccinellids. The experiments demonstrated the importance of extrafloral nectar in delaying starvation and preserving the fecundity of the otherwise predatory beetles, during periods when prey is scarce (Lundgren and Seagraves 2011).

Extrafloral nectaries are known to attract a diversity of ant species as well, which can form mutualistic relationships with the plant through the exclusion of herbivores (Koptur 1992, Stephenson 1982, Suzuki *et al.* 2004). On *V. faba*, three species of ants have previously been frequently observed feeding on the extrafloral nectar (Table 1.2), whereas several other ant species including *Camponotus japonicas* Mayr, *Formica japonica* Motschulsky, *Paratrechina sakurae* Ito, and *Pristomyrmex pungens* Mayr have been observed elsewhere on the plant (Katayama and Suzuki 2003). Although ants foraging for extrafloral nectar can be beneficial in reducing herbivory, their presence can also be antagonistic with other nectar seeking visitors, such as parasitoid wasps, causing the benefits of ants and parasitoid wasps to be non-additive (Cuautle and Rico-Gray 2003). Ants can also antagonize pollinators, such as bees (Junker *et al.* 2007), and some researchers have theorized that EFNs may be used to lure ants away from floral nectaries in order to avoid this antagonism (Marazzi *et al*. 2013). However, recognition of ants by pollinators does not always appear to lead to pollinator avoidance of the flowers. The presence of decoys shaped like aggressive ant species on *Heteropterys pteropetala* A. Juss. has

resulted in decreased fruit set compared to circular decoy controls, despite actual ants under the same conditions having no significant effect on fruit set (Assunção *et al.* 2014). Only the more aggressive ant species have been found to negatively impact pollination when present on plants producing extrafloral nectar (Ness 2006). In general, ants are known to search more frequently and with a greater persistence for resources which are renewed regularly (Fourcassié and Traniello 1994), and EFNs are known to be able to rapidly replenish their nectar (Gaffal 2012). As such, frequent visits to extrafloral nectaries, as well as searching behaviour nearby may contribute to the ability of ants to ward off both insects which are detrimental, and those that could be beneficial to the host plant.

Despite repelling various insects, ants are also known to sometimes protect colonies of aphids (Engel *et al.* 2001), which can result in the ants having an overall detrimental effect to plants, including *V. faba* (Oliver *et al.* 2007). The relationship between the ants and aphids can be complex, with the ants benefitting or harming the aphid populations to different degrees depending on the species of ant and aphid (Sakata and Hashimoto 2000). Other important factors in the ant-aphid relationship include the aphid's population size and the properties of the sugar produced by the aphids (Katayama and Suzuki 2003). Therefore, although ants may protect extrafloral nectar secreting plants from most herbivorous insect species, the effect of ants on other insects associated with the plant can dramatically alter their relationship with the plant under certain circumstances.

2.1.2 Pollinators of *V. faba*

Records of *V. faba*'s pollinators are highly variable between studies, with one study reporting nearly all visitors were honeybees (92%) and bumblebees (almost 8%) (Kołtowski 1996c). In contrast, other studies have found wild bees were the predominant floral visitors and appear to be the most effective pollinators (Benachour *et al.* 2007, Bond and Kirby 1999). This discrepancy is due to *V. faba*'s floral visitors varying greatly between locations (Pierre *et al.* 1999), so the floral visitors present in a specific region of Saskatchewan, Canada may differ from past studies. Even studies within the same country report variations in both which species were present, as well as which were dominant (Aouar-sadli *et al.* 2008, Benachour *et al.* 2007). The roles of different bees may also vary by both species and region, as *Apis mellifera* has been found to rob nectar only when holes have already been chewed through the base of the flower by

Bombus spp. (Benachour *et al.* 2007, Free 1962). Thus far, the most abundant floral visitors known from *V. faba* come from the hymenopteran family Apidae (Table 1.1), which includes *Anthophora plumipes* and *Apis mellifera*, as well as species of *Bombus* and *Eucera* (Aouar-sadli *et al.* 2008, Benachour *et al.* 2007, Bond and Kirby 1999, Kendall and Smith 1975, Kołtowski 1996c, Pierre *et al.* 1996). There is also a diversity of other less frequent visitors, and a number of species that have been found only rarely at *V. faba*'s flowers (Table 1.1). Past studies have examined *V. faba*'s potential pollinators in multiple countries, including Algeria (Aouar-sadli *et al.* 2008, Benachour *et al.* 2007), Canada (Malaipan 1979), Denmark (Poulsen 1973), Egypt (El-Berry *et al.* 1974), France (Pierre *et al.* 1996, Tasei 1976), Poland (Kołtowski 1996c), the United Kingdom (Bond and Kirby 1999, Free 1962, Kendall and Smith 1975), and the United States of America (Nuessly *et al.* 2004). This study seeks to explore any differences in the identity and abundance of insects that visit the flowers and EFNs of cultivars with or without tannins. The relative locations of the visited EFNs will also be examined, as the presence of tannins may result in the increased visibility of EFNs near the plant's apex.

2.2 Materials and methods

2.2.1 Study site

To study the insects attracted to the floral and extrafloral nectaries of *V. faba*, several cultivars were grown in two outdoor locations for the first year of the study: the University of Saskatchewan's Biology Research plot (52.132°N, 106.634°W), and a field near the University of Saskatchewan's Crop Science Field Laboratory (52.136°N, 106.622°W). In the second year, only the Biology Research plot was used, in order to focus on the interactions of ants with *V. faba.* At both locations, seeding occurred on two dates, to allow sampling to continue into the fall, after the first planting no longer produced extrafloral or floral nectar. These two crops of *V. faba* at the Biology Research plot were contained in two, approximately 9 x 9 m areas, while the remainder of the plot contained a wide variety of other research, ornamental, and garden plants. Plants adjacent to *V. faba* that were flowering during the course of the study included hollyhocks (*Alcea* sp.), sunflowers (*Helianthus* spp.), sweet peas (*Lathyrus odoratus* L.), bee balm (*Monarda* sp.), beans (*Phaseolus vulgaris* L.), and corn (*Zea mays* L.). The Crop Science field was located adjacent to the remainder of the University of Saskatchewan's test plots and relatively extensive agricultural areas, and contained many additional cultivars of *V. faba* not

grown as a part of this study, as well as lentils (*Lens culinaris* Medik.), potatoes (*Solanum tuberosum* L.), and other crop plants.

Seeds of *V. faba* were obtained from Dr. Albert Vandenberg and Mr. Brent Barlow of the University of Saskatchewan's Department of Plant Sciences. A randomized complete block design enabled a comparison between the *V. faba* cultivars, and each block included two tannin-rich cultivars with dark-coloured EFNs (Fatima and SSNS-1), and two tannin-free cultivars with colourless nectaries (Snowbird and Snowdrop). Blocks were set up similarly to the design of Nuessly *et al.* (2004) and Bugg *et al.* (1989), although the individual blocks and number of blocks were smaller due to space limitations. To allow for potential germination failures, each of the five blocks featured four patches, one for each of the four chosen varieties. Patches consisted of 20 or more plants each, with excess plants removed to produce an equal number of plants/cultivar/block, to eliminate the effect of uneven germination rates among patches. Final patch size was 16 plants per patch, with a total of four blocks per plot being used. Soil within the plots, as well as in the surrounding areas, was tilled earlier in the spring, prior to seeding. Seeds of *V. faba* were first planted on May 23, 2013, and on June 2 in 2014, with a second set planted around 45 days later (McVetty *et al.* 1986). In the second year (2014), the plot was re-seeded on June 27, as unusually wet weather caused the first set of seeds to rot. A second plot of *V. faba* was seeded July 16, 2014, such that nectar production was continuous throughout the summer and fall, between the two successive groups of plants. No fertilizers or herbicides were used throughout the growing seasons, and weeds were controlled through manual tilling of the soil, although mechanical tilling occurred within a meter of the Crop Science plot. On August 27, 2013, the pesticide Orthene (1.2g/L) was applied using a hand-held sprayer to the area surrounding, but not including, the Crop Science plot. Following the pesticide application, data from the Crop Science plot was excluded from the study.

2.2.2 Surveys of nectary visiting insects

Throughout the period when the EFNs were functional and flowers were present, timed periods of monitoring and capturing insects took place at each block during the summer of two consecutive years (2013, 2014). EFN surveys occurred throughout growth stages 1-8 (leaf development to fruit ripening) of *V. faba* (Lancashire *et al.* 1991, Weber and Bleiholder 1990), until the EFNs ceased functioning. Flower surveys were conducted while 50% of *V. faba* within

a patch were flowering (growth stage 6). Individual surveys for nectar drinking insects were one hour long per patch, with all four patches in a block being surveyed consecutively. Initial 2013 survey periods took place throughout many different times of the day in order to capture potential diel patterns; however, visitors were most often caught in the hours around midday, and were not as active in the early morning or late in the afternoon. Sampling was therefore focused around this period, with block surveys taking place from around 8:30am to 12:30pm, and 12:30pm to 4:30pm. Cultivars were sampled at different times on different days, to negate the effect time of day may have had on insect visitation to different cultivars. Surveys primarily took place from the start of July until the end of August, with a few surveys conducted in September. In 2014, 96 one-hour surveys took place, consisting of four replicate surveys for the four cultivars in each of the four plots of the first set of plants, and two replicate surveys per cultivar per block for the second set of plants. Sampling effort in 2013 was similar, although it was split between two locations (Crop Science field and Biology Research plot).

Insects were captured through the use of an insect net and aspirator, and were placed into separate re-sealable bags with the date, time, block, and cultivar noted, then subsequently euthanized through freezing. The position of the visited EFN's node was also recorded for insects captured in 2013, with the nodes of *V. faba* numbered from the bottom of the plant upward. In 2014, the position of the EFN's node was recorded relative to both the bottom of the plant, and to the most distal pair of stipules which did not overlap with the EFNs on the set of stipules above it. The relative position of the latter EFNs, herein referred to as the Primary Active EFNs (Fig. 1.1a), was used to approximate the location of the pair of EFNs producing the most nectar, which were consistently observed to be just below the cluster of overlapping stipules near the apex of the plant. The node at which the Primary Active EFNs were located shifted upward as the plant grew, as EFNs produced differing amounts of nectar throughout the plants' life. This resulted in the Primary Active EFNs staying at the same relative position on the continuously growing plants, despite each pair of EFNs remaining at approximately the same absolute height from the ground following their time as the Primary Active EFN. The EFN's relative location on the plant was recorded for up to the first ten insects observed visiting an EFN during each 2014 survey, regardless of whether or not the insects were captured. Captured insects were later pinned, and all insect visitors to *V. faba* were identified to family, genus, and species whenever possible. Identification took place using dichotomous keys for insects

including *Bombus* (Stephen 1957, Williams *et al.* 2014), Diptera (Shewell 1939, Triplehorn and Johnson 2005), Formicidae (Fisher and Cover 2007, Glasier *et al.* 2013), Hemiptera (Kelton 1980) Ichneumonidae (Cresson 1868, Fitton *et al.* 1988, Gauld and Wahl 2013, Goulet and Hubert 1993, Heinrich 1960, 1961, Tereshkin 2009, Townes and Townes 1960), and Vespidae (Buck *et al.* 2008, Carpenter and Glare 2010, Kimsey and Carpenter 2012).

2.2.3 Statistical methods

Generalized mixed models were used to determine the effect of tannins on insect visits to the EFNs and flowers of different *V. faba* cultivars. Statistical models used the presence/absence of an insect species as the response variable, cultivar as an explanatory variable, and a variety of random variables, including whether plants belonged to the first or second set of *V. faba* seeded in a year (Plot), the survey year (Year), which block the patch of *V. faba* was contained in (Block), and when the survey took place relative to other surveys (Survey). The random variables were used to account for some of the variation in insect presence/absence, and were nested within one another (Plot/Year/Block/Survey) to produce the best-fitting model. The generalized mixed models for EFN visitors accounted for the binary distribution of the data, whereas models for floral nectary visitors factored in the data's Poisson distribution.

Of the visitors to EFNs, only ant species found at the Biology Research site were examined using generalized mixed models, as data from other extrafloral nectar consuming insect species consisted almost entirely of absences from the surveys (Bates *et al.* 2014). Likewise, generalized mixed models of only the two most common floral nectary visitors were created. Although floral nectary visitor data was recorded during all surveys, only specific floral nectary survey data was included in statistical comparisons between cultivars. Floral nectary surveys occurred when 50% or more of the plants of each cultivar in a block were flowering, as flowering did not begin or end at the same time for all cultivars.

Successful models were selected through comparisons of Akaike's Information Criterion (AIC) values. Goodness-of-fit Chi-squared tests ($p < 0.05$) were used to ensure the selected models had greater explanatory power (lower residual deviance) and lower AIC values than the respective null models. Post-hoc analysis consisted of orthogonal contrasts that used single coefficients to determine where the differences within factors arose, such as which cultivars were different from one another. Variance components analyses were also used to examine the

generalized mixed models for each ant species, and were able to provide the proportion of explained variance attributable to each of the random factors. All model selection and post-hoc analyses in this study were accomplished using the R statistical program (R Core Team 2015). The specific *p*-values for various comparisons are detailed in Appendix 1 alongside AIC values and models' residual deviances (Tables A.1, A.2). Holm's sequential version of the Bonferroni correction for *p*-values [(C - *i* +1) * *p*] was applied to those values resulting from orthogonal contrasts (Holm, 1979), which were used to differentiate the cultivar categories present in bar graphs.

To examine the relationship between the relative position of EFNs and the species of insects that had visited them, contingency tables were each analyzed using a Chi-squared test of independence (Venables and Ripley 2002). Infrequently visited EFNs were combined, with one category for EFNs three or more nodes below the Primary Active EFN, and another for nodes two or more above the Primary Active EFN. Two insect species visited frequently enough to analyze in separate categories in the Chi-squared test, whereas the remaining species were grouped together in a third category. A Chi-squared test was also used to compare the cultivar of *V. faba* grown and the relative position of the visited EFN on the plant, using the same categories for the EFN positions as the previously mentioned Chi-squared test. The threshold for the tests' statistical significance was defined using *p*-values ($p < 0.05$).

2.3 Results

2.3.1 Insect visits to extrafloral nectaries

A variety of adult insects were found feeding at the extrafloral nectaries on the stipules of *V. faba*, including ants (Fig. 2.1a), beetles (Fig. 2.1b), flies, wasps (Fig. 2.1c,d) and a plant bug (Table 2.1). However, the vast majority of insects found drinking extrafloral nectar were ants, which were often so numerous within patches of plants that the rate of visitation per plot could not be recorded due to multiple visits occurring simultaneously. This abundance of EFN visiting ants was in stark contrast to all other visiting insect species, most of which were encountered feeding at the EFNs only once. One fly species (*Camptoprosopella borealis*) imbibed extrafloral nectar in 18 of 96 surveys in 2014, but no other species, aside from ants, was encountered at EFNs even 10 times throughout the two years of surveys. Ants were also the only species to consistently consume extrafloral nectar while being present in plots of *V. faba* (Fig. 2.1a).

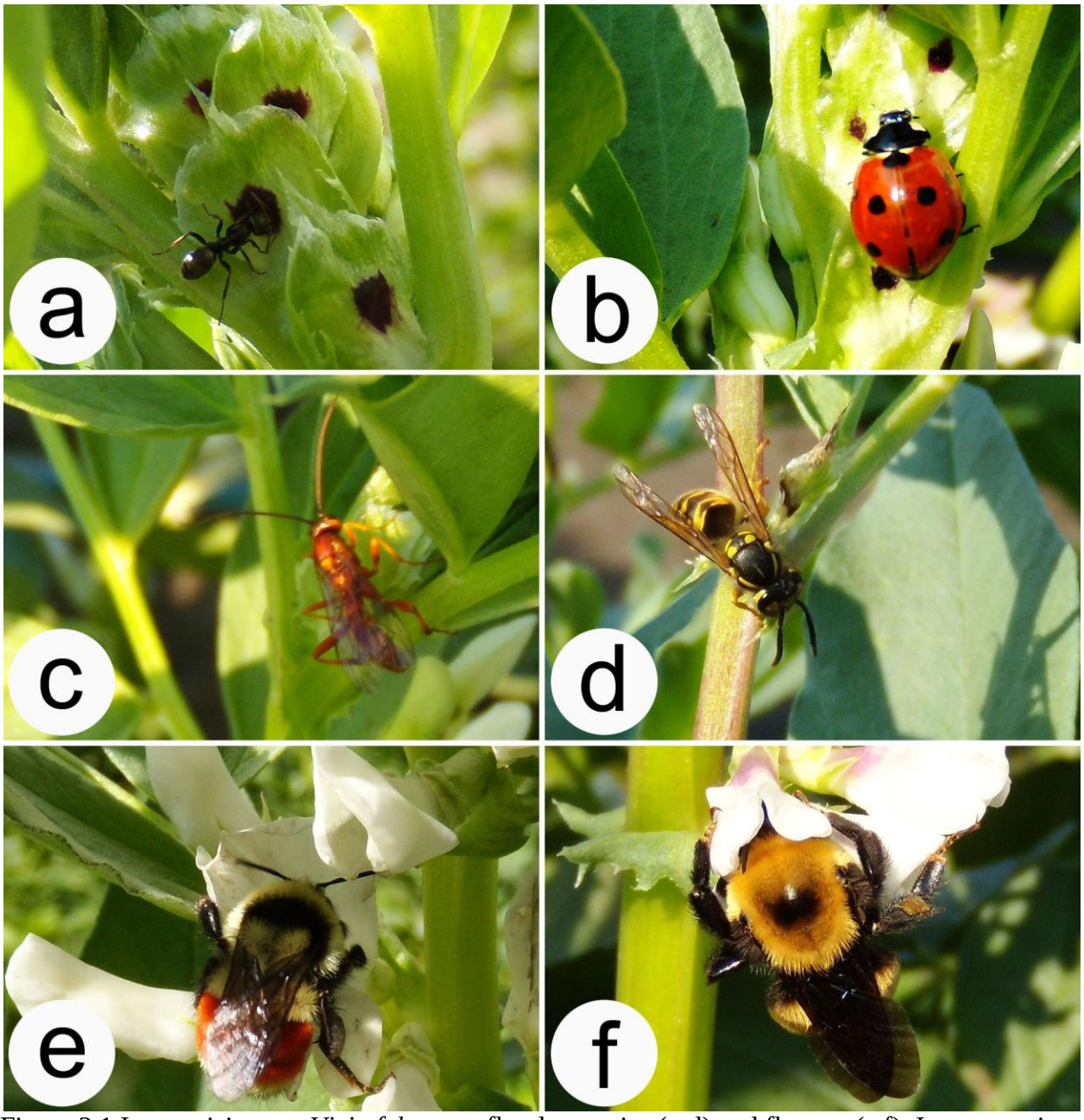


Figure 2.1 Insect visitors to *Vicia faba* extrafloral nectaries (a-d) and flowers (e,f). Insect species are a) *Formica podzolica*, b) *Coccinella septempunctata*, c) *Banchus flavescens*, d) *Vespula germanica*, e) *Bombus huntii*, and f) *B. nevadensis*. Both tannin-containing (a,b,d,f) and tannin-free (c,e,) cultivars are depicted.

Camptoprosopella borealis was often as common as ants within the plots, but would only occasionally consume nectar.

Additionally, numerous ichneumonids were seen passing through the study areas, often exhibiting searching behaviour amongst the *V. faba* vegetation before leaving; their consumption

of nectar was rare (Fig. 2.1c). Predatory wasps (Vespidae) were also encountered hunting for prey far more often than they were found consuming extrafloral nectar (Fig. 2.1d). However, wasps' visits to the EFNs were still much more common than visits by any hemipterans, including the plant bug *Lygus lineolaris*, as well as far exceeding the number of visits by fly species other than *C. borealis*. Insects such as *L. lineolaris* and anthomyiid flies were encountered during almost every survey, but only once each were they found consuming extrafloral nectar. Species of Coccinellidae were also found at EFNs, including *Coccinella*

Table 2.1 List of insect visitors found attempting to consume the nectar of *Vicia faba* in Saskatoon, SK, 2013-2014.

Nectary Location	Order	Family	Genus or Species and Authority
Flower	Diptera	Syrphidae	-
	Hymenoptera	Apidae	*Apis mellifera* Linnaeus
			Bombus borealis Kirby
			B. huntii Greene
			B. nevadensis Cresson
			B. perplexus Cresson
			B. terricola Kirby
		Halictidae	-
		Vespidae	*Ancistrocerus albophaleratus* (de Saussure)
	Lepidoptera	Pieridae	*Pieris rapae* (Linnaeus)
Stipule	Coleoptera	Coccinellidae	*Coccinella septempunctata* Linnaeus
			Cryptolaemus montrouzieri Mulsant
			Hippodamia tredecimpunctata (Linnaeus)
	Diptera	Anthomyiidae	-
		Calliphoridae	*Phormia regina* (Meigen)
		Lauxaniidae	*Camptoprosopella borealis* Shewell
		Sarcophagidae	-
		Tachinidae	*Hystricia abrupta* (Wiedemann)
	Hemiptera	Miridae	*Lygus lineolaris* (Palisot de Beauvois)
	Hymenoptera	Formicidae	*Formica lasioides* Emery
			F. neoclara Emery
			F. podzolica Francoeur
			Lasius pallitarsis (Provancher)
			Myrmica brevispinosa Wheeler
		Ichneumonidae	*Banchus flavescens* Cresson
			Ichneumon annulatorius Fabricius
			Pimpla pedalis Cresson
		Vespidae	*Dolichovespula arenaria* (Fabricius)
			Vespula germanica (Fabricius)

septempunctata (Fig. 2.1b), which was observed consuming extrafloral nectar several times, although both *C. septempunctata* and *Hippodamia tredecimpunctata* were present on *V. faba* far more often than they were present at the EFNs. Larvae of both coccinellid species were also frequently present on *V. faba*; however, they did not appear to feed at the EFNs during any of the surveys.

Due to the infrequent visits to EFNs by insects other than ants, diversity values could not be calculated. With two plots of *V. faba* planted at two locations during 2013, and two plots at a single location the next year, there was a total of six year/location/plot combinations, with four of them receiving a maximum of two non-ant EFN visitors each. Of the remaining two year/location/plot combinations, the earlier planted Biology plot in 2014 had *V. faba* EFNs visited 20 times by flies, most of which were *C. borealis*. The first Crop Science plot to be planted in 2013 had 29 non-ant EFN visits during survey times, comprised mostly of parasitoid wasps (Ichneumonidae, n = 7), predatory wasps (Vespidae, n = 12), and predatory beetles (Coccinellidae, n = 6), with only three visits by flies, and one by a hemipteran. Cultivars Fatima, SSNS-1, Snowbird, and Snowdrop received 10, 7, 11, and 1 visit(s), respectively. Throughout the entire study, there were no insect species observed consuming extrafloral nectar that also consumed floral nectar, resulting in no overlap between the two sections of Table 2.1.

2.3.1.1 Ants and extrafloral nectaries

The relative frequency of visits to EFNs was much greater for ants compared to other insects, and as a result, extrafloral nectar preferences between *V. faba* cultivars with and without tannins were examined for each ant species encountered. Using the presence or absence of extrafloral nectar consuming ants from plots of *V. faba* (n = 144), generalized mixed models were evaluated based on the model's fitness. Null models were compared to models using cultivar as an explanatory variable; however, including the plants' cultivar did not always improve model fitness. For both *Formica lasioides* and *F. neoclara*, there were only relatively minor differences between cultivars regarding how many surveys the ant species was found consuming extrafloral nectar (Fig. 2.2a,b). Goodness-of-fit Chi-squared tests showed no significant difference between the null models, and those with cultivar as an explanatory factor (Table A.1). Additionally, there was no significant difference between the AIC values of the two models for *F. lasioides*, whereas the null model for *F. neoclara* produced a significantly lower

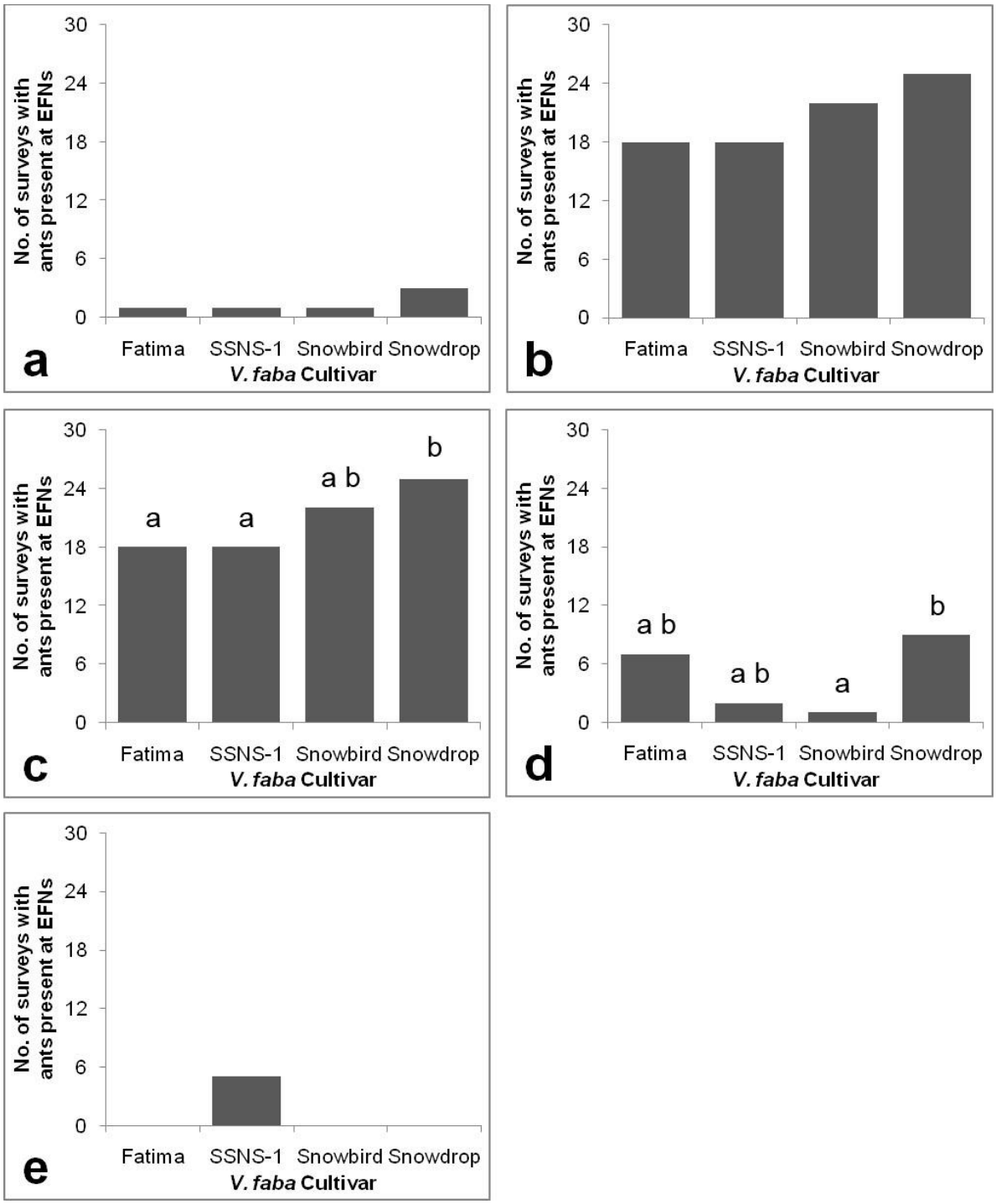


Figure 2.2 Frequency distributions of the number of surveys with an ant species present at the extrafloral nectaries for different cultivars of *Vicia faba*. Matching letters indicate cultivar and node combinations that are not statistically different from one another ($p > 0.05$), although data quantity limited statistical testing to only two of the graphs (c,d). Data are arranged per ant species for visits recorded to tannin-containing varieties (Fatima, SSNS-1) and tannin-free varieties (Snowdrop, Snowbird) by a) *Formica lasioides*, b) *F. neoclar*a, c) *F. podzolica*, d) *Lasius pallitarsis*, and e) *Myrmica brevispinosa*. All surveys took place in the University of SK Biology research plot from 2013 - 2014.

AIC value than that of the model including cultivar, indicating that the inclusion of cultivar lowered the model's fitness.

The cultivar of *V. faba* did produce significant differences between the model with and without it as an explanatory factor in the Chi-squared test for *F. podzolica*, and the model with cultivar had a significantly lower AIC value (Table A.1). The remaining unexplained deviance for the model including cultivar was also lower than the null model, indicating that cultivar was a useful explanatory variable in the model. Orthogonal contrasts were then used during post-hoc testing of the model (Table A.2). The results showed some statistical differences in extrafloral nectary visits between cultivars for *F. podzolica*, which did somewhat correspond to differences in tannin presence/absence (Fig. 2.2c). The cultivars Fatima and SSNS-1 were not statistically different, whereas cultivar Snowdrop differed from both the former cultivars. However, the cultivar Snowbird was not statistically different from any of the other cultivars. The differences between cultivars appeared fairly similar in magnitude, but with the pattern reversed compared to *F. neoclara*, which more frequently visited tannin-producing cultivars (Fig. 2.2b).

A Chi-squared test comparing the *Lasius pallitarsis* null and explanatory models also revealed a statistical difference between the two generalized mixed models. The model using cultivar as an explanatory variable had a significantly lower AIC value, as well as reduced residual unexplained variance compared to the null model (Table A.1). Differences between cultivars were therefore examined with post-hoc testing, using orthogonal contrasts. Cultivars did not group by tannin presence/absence, as Snowbird and SSNS-1 had more similar results than the two tannin-free cultivars had with one another (Fig. 2.2d, Table A.2).

Generalized mixed models of *Myrmica brevispinosa* were also compared to determine if including cultivar as a variable increased the model's fitness. Results of the Chi-squared test showed a significant difference between the two, with a decrease in AIC and residual deviance in the model including cultivar, indicating the model's improved fitness (Table A.1). However, the total number of surveys where EFNs were visited by *M. brevispinosa* was not only low (Fig. 2.2e), but aside from a single survey, the ant was detected consuming extrafloral nectar in only one patch per year. Due to the highly limited foraging area recorded for *M. brevispinosa*, post-hoc testing produced errors in the statistical program, and therefore orthogonal contrasts of different *V. faba* cultivars could not be included in the analysis.

Overall, there was an average of slightly more than one ant species found visiting EFNs during each one-hour survey of a *V. faba* patch. The average number of different ant species to visit *V. faba* EFNs during an individual survey (n = 36 per cultivar) for Fatima, SSNS-1, Snowbird, and Snowdrop ± the standard error of the mean (S.E.M.) was 1.1 ± 0.1, 1.1 ± 0.1, 0.9 ± 0.1, and 1.3 ± 0.2, respectively.

Variance components analyses were also used to analyze the factors influencing EFN visits by ants. Only the variance explained by the random factors used in the generalized mixed models was examined by the analyses (Table 2.2). All random factors used in the generalized mixed models improved the ability of the models to reduce the residual variance for at least one ant species.

Table 2.2 Proportions of explained variance (%) from variance components analyses of generalized mixed models of ant species presence at *Vicia faba* extrafloral nectaries.

	Ant species				
Random factors	*Formica neoclara*	*Formica lasioides*	*Formica podzolica*	*Lasius pallitarsis*	*Myrmica brevispinosa*
Plot	83.0	0.0	0.0	0.0	0.0
Year	3.0	0.1	74.8	61.2	0.0
Block	14.0	0.2	25.2	38.8	100.0
Survey	0.0	99.7	0.0	0.0	0.0

2.3.1.2 Extrafloral nectary relative position

Another factor which varied between ant species was the relative position of the EFNs visited by the ants. When a Chi-squared test of independence was performed on a contingency table of the visited nodes, the null hypothesis that the species of insect visiting was independent of the relative position of the node visited was rejected (Degrees freedom = 10, $p = 0.006$). In spite of the large number of observations recorded (n = 931), the majority of species did not have enough data to be analyzed separately without compromising the analyses, as the majority (62%) of the visits to EFN positions were recorded from *F. podzolica*, and another 24% from *F. neoclara*. The remainder of the visits with EFN position recorded in 2014 came from six of the other species listed in Table 2.1 (*C. borealis, D. arenaria, F. lasioides, L. pallitarsis, M. brevispinosa,* and *V. germanica*), and were placed in a single category for the purposes of the analyses. However, despite the differences between species noted by the Chi-squared test, the

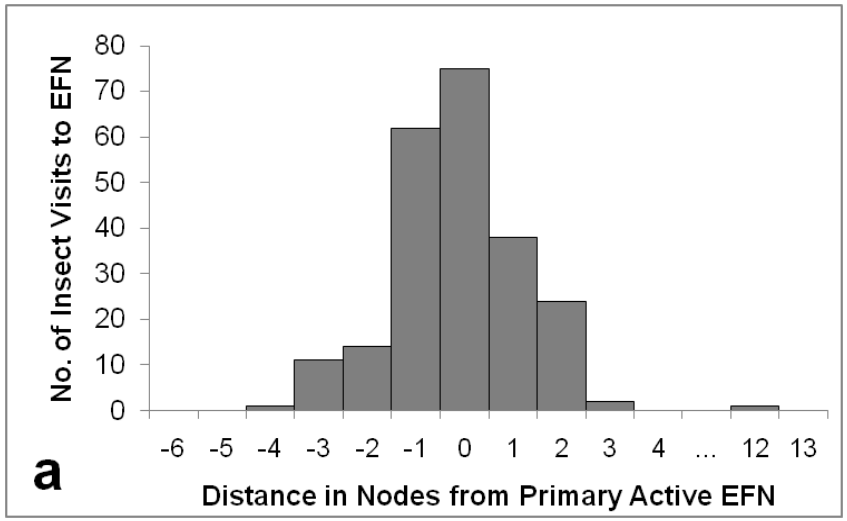
average relative position of the node visited by the different insects varied by, at most, half of a single node between all the categories (Fig. 2.3a-c).

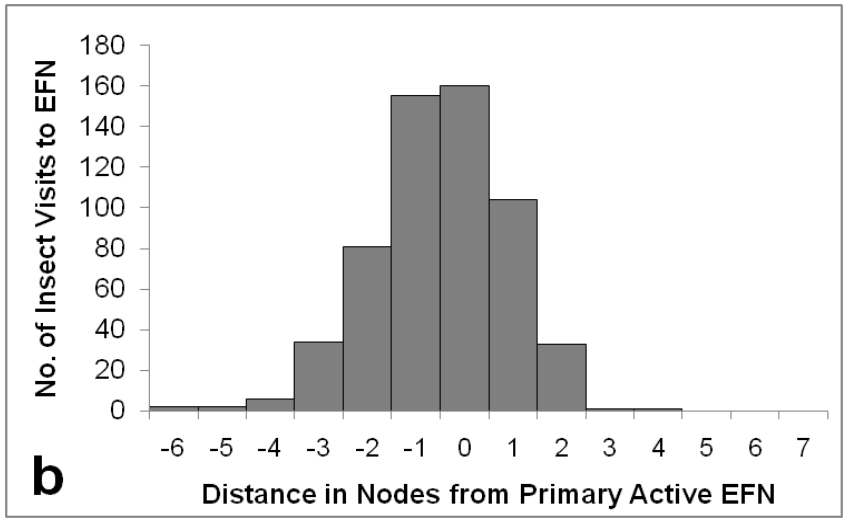
The relative positions of visited EFNs were also compared to the cultivar of *V. faba* that the EFN belonged to, and the Chi-squared test suggested the two variables were independent. The average node visited was highly conserved between cultivars, and varied by less than 0.2 nodes (Fig. 2.4a-d). As well, the observations were far more evenly distributed across cultivars than for the different insect species, as the proportion of visits received by the four cultivars was between 24% and 26% each. Of the recorded visits to the extrafloral nectaries, 98% occurred within three nodes of the Primary Active EFN, with 30% of the visits belonging to the Primary Active EFN itself. In contrast, there was an average of 11 nodes closer to the plants' base than the Primary Active EFN, with a range of 1 to 28 nodes more basal to it throughout the season's observations. The average of the visited node numbers was -0.4, indicating a slightly greater number of ants visited EFNs basal to the Primary Active EFN than apical to it.

2.3.2 Insect visits to floral nectaries

The floral nectaries of *V. faba* were visited by a variety of insects during the study, most of which belonged to the family Apidae (Table 2.1). In 2013, 74 % of visitors were species of *Bombus* (Fig 2.1e,f), whereas all of the visitors observed during 2014 surveys were species of Apidae, including the genera *Apis* and *Bombus* (Fig. 2.5a,b). In addition to apid bees, a single halictid bee was also observed attempting to consume nectar from a flower, along with a single syrphid fly and vespid wasp (Table 2.1). Overall, the rate of insect visits to *V. faba* flowers was quite low at 0.03 visitors/hour/plant, which amounted to approximately one visitor for every two hours spent surveying a 16-plant patch. Antagonistic interactions between ants and potential pollinators such as bees were not observed, aside from a single incident where a *Bombus ternarius* Say retreated after *Formica podzolica* attempted to remove the bumblebee from a leaf of *V. faba*.

In contrast to the models of EFN visitors, cultivar was not significant as an explanatory factor in any of the models of insect visits to nectaries during the floral nectary surveys. The model of visits by *Bombus nevadensis* had a significantly higher AIC value when cultivar was included compared to the null model (Table A.1); consequently, the null model was retained. The model for *Apis mellifera* had similar AIC for both null and explanatory models, and no





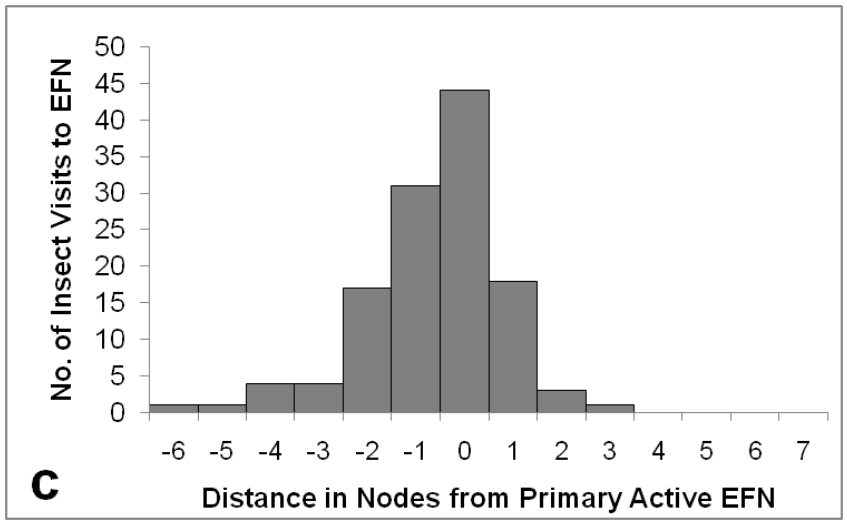


Figure 2.3 The frequency of visits to extrafloral nectaries (EFNs) of different relative positions on the stem of *Vicia faba* by different insects: a) *Formica neoclara*, b) *F. podzolica*, and c) All other spp. The EFN's position was defined as its distance in nodes away from the Primary Active EFN. Negative numbers indicate a position of the node on the stem basal to the Primary Active EFN, and positive numbers indicate a position apical to it. The presented data was collected during the summer of 2014.

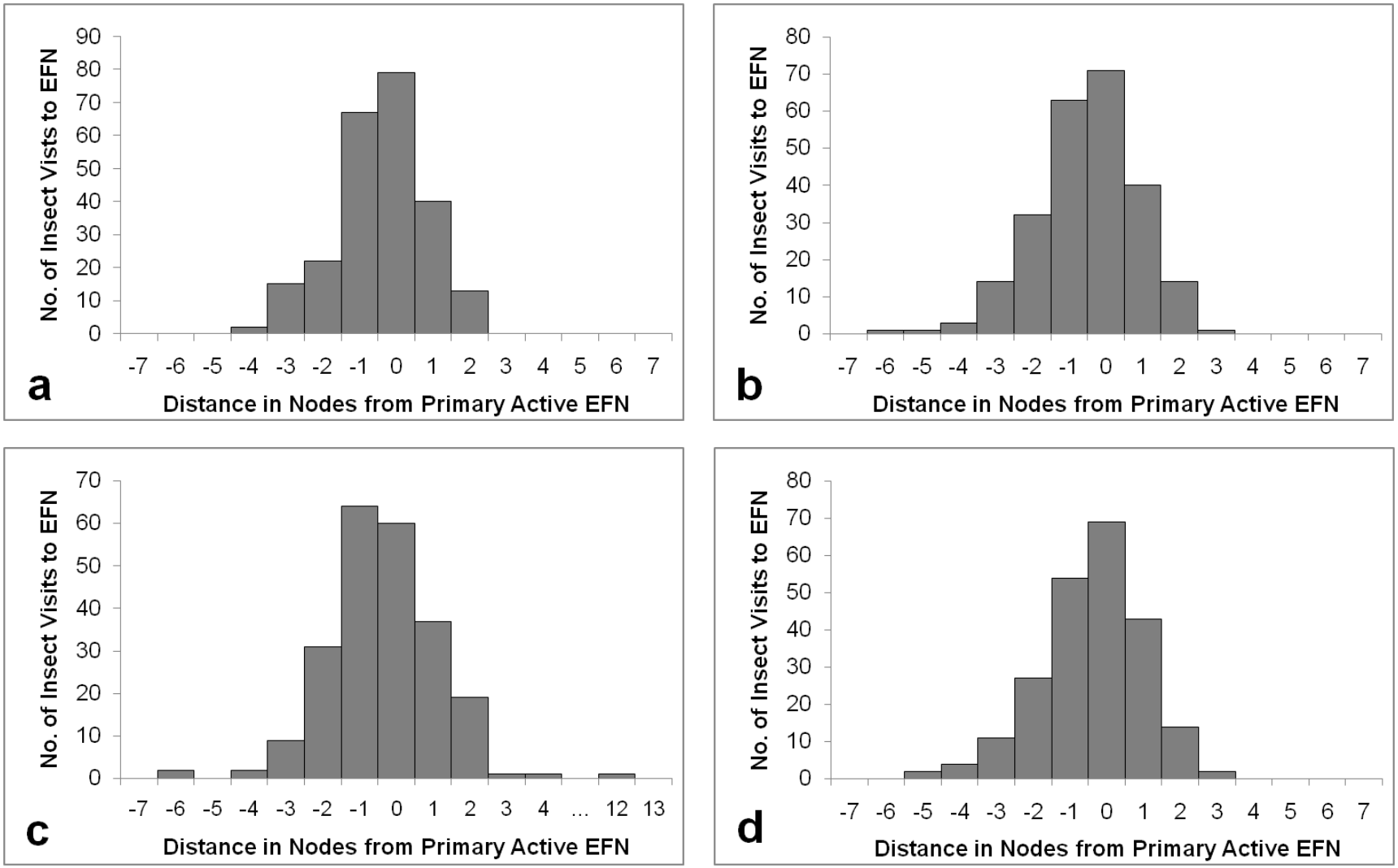


Figure 2.4 The frequency of insect visits to extrafloral nectaries (EFNs) of different relative positions on the stem of *Vicia faba* cultivars a) Fatima, b) SSNS-1, c) Snowbird, and d) Snowdrop. The EFN's position was defined as its distance in nodes away from the Primary Active EFN, which has been assigned the value, 0. Negative numbers indicate a position of the node on the stem basal to the Primary Active EFN, and positive numbers indicate a position apical to it. The presented data was collected during the summer of 2014.

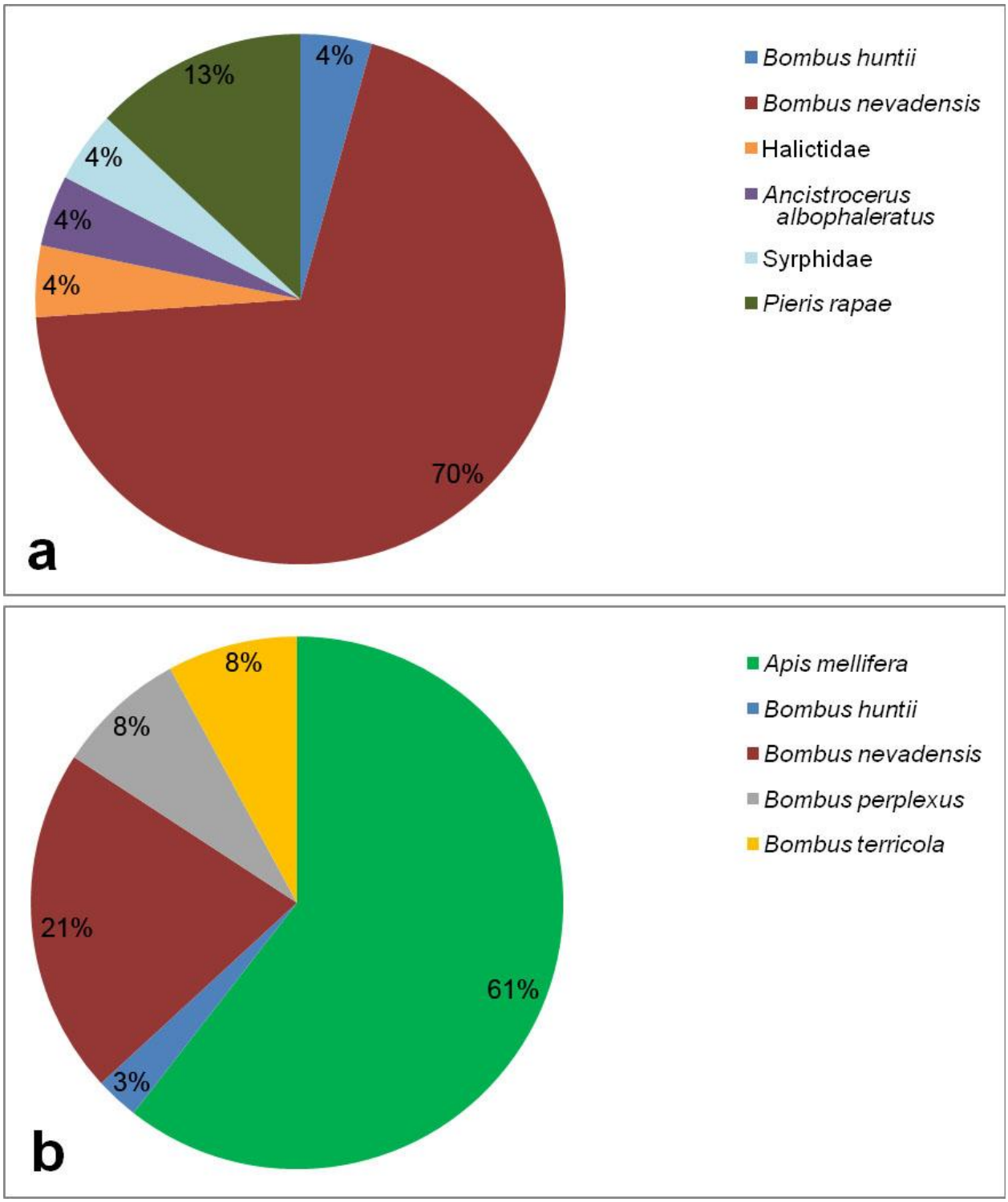


Figure 2.5 Proportions of different insect taxa recorded on *Vicia faba* flowers during floral nectary visitor surveys at the Biology Research plot (Saskatoon, SK) in a) 2013 (n = 23) and b) 2014 (n = 38).

significant difference between the two models. The null model was therefore not rejected. Chi-squared tests between the two sets of null and explanatory models did not show a significant difference in explanatory power between the pairs of models (Table A.1). With only nine species visiting *V. faba* flowers during the two years of surveys, the overall species richness of floral nectary visitors was low, with cultivars varying from only three different species visiting Snowdrop, to six species visiting SSNS-1. Four species were found at flowers of cultivars Fatima and Snowbird during the floral nectary surveys. Total visitor numbers were also low, and the majority of species were not present in most year/location/cultivar combinations. Diversity numbers therefore could not be calculated, with each cultivar receiving between six and 12 visits in total during the flower surveys, which were fairly evenly dispersed throughout the two years and locations.

Local weather conditions were not as similar between years, with higher spring (May) temperatures and lower precipitation during the first year of the study (2013), compared to relatively wet conditions of 2014 (Fig. 2.6a,b).

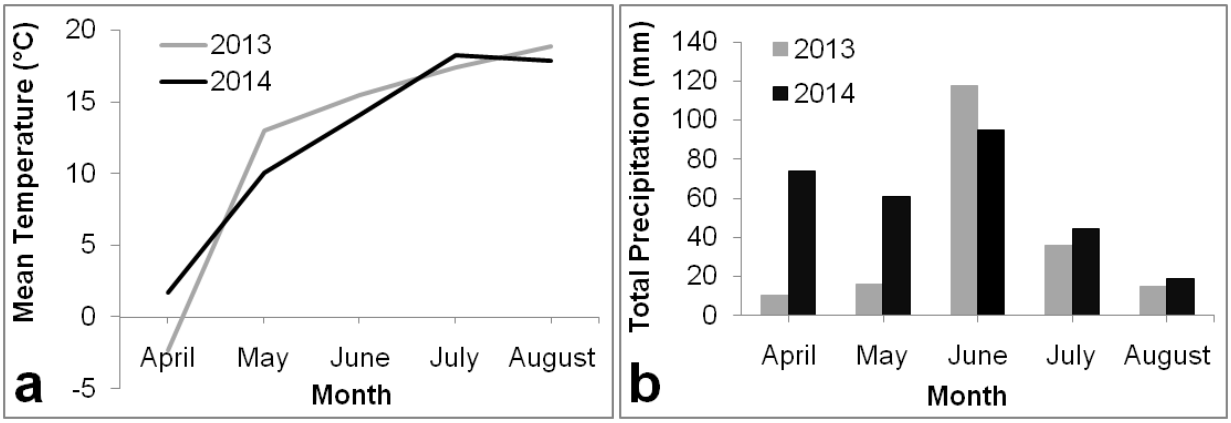


Figure 2.6 Weather data for April to August of 2013 and 2014, including a) mean temperatures (°C) and b) total precipitation (mm). Data was recorded in Saskatoon at the John G. Diefenbaker International Airport by the Government of Canada (2016).

2.4 Discussion and conclusions

2.4.1 Factors affecting insect visitation to extrafloral nectaries

Overall, the diversity of insects found consuming extrafloral nectar was comparable to other studies of *V. faba*; however, a number of the encountered insects (Table 2.1) were not previously recorded as visitors to the EFNs (Table 1.2). One such insect was *Camptoprosopella borealis*, the highly abundant lauxaniid fly which was found flying near, and resting on, the

stems and leaves of *V. faba*. The adult flies consumed extrafloral nectar on occasion, while taking shelter from the wind and sun. However, their abundance in the area was likely due to the availability of large amounts of decomposing organic matter, upon which the flies' larvae feed (Knodel 2013, Miller 1977). High levels of organic matter were produced as a result of periodic manual weed control. Although the flies may not have been previously associated with *V. faba*, they are known to sometimes be common in wheat fields, as well as other crops such as corn and soybean. Other fly species were seen consuming extrafloral nectar no more than once each, despite some of the species' prevalence amongst the *V. faba*. All the EFN visits by the flies, as well as the single occurrence of a *Lygus lineolaris* consuming extrafloral nectar, did not appear to be due to the insect deliberately searching out the nectar. Rather, the insects were often found to land or crawl on various parts of the plant, and would only drink nectar when they already happened to be on a stipule. Even once the flies were on a stipule, nectar consumption occurred infrequently, in contrast to Hetschko's (1908) study which found several species of flies occurred frequently at the EFNs, and a species of *Lygus* was recorded as not rare at *V. faba* EFNs in what was previously Teschen, Austria.

Ichneumonid wasps were quite uncommon throughout the surveys, with only three species found consuming extrafloral nectar (Table 2.1). Of the three species, only *Banchus flavescens* was observed at the EFNs (Fig. 2.1c) in more than one instance. The relative absence of parasitoid wasps was similar to the studies of *V. faba* in both Manitoba (Malaipan 1979) and Florida (Nuessly *et al.* 2004), in contrast to the numerous Ichneumonidae species reported in Massachusetts (Bugg *et al.* 1989) (Table 1.2). Although the presence of ants is known to sometimes discourage other EFN visiting insects (Cuautle and Rico-Gray 2003), wasp numbers were still low in those surveys of *V. faba* which were free of ant colonies. The reason for such a discrepancy in ichneumonid numbers between studies is unknown, and could be dependent on environmental factors such as climate and the local vegetation available to the wasps.

Compared to the parasitoid wasps, predatory wasps visited EFNs approximately twice as often, and were also found flying near *V. faba* far more often. Several species of vespid (yellowjacket) wasps were common in the study areas, but only two species were observed to consume extrafloral nectar, which mainly occurred towards the end of the growing season. The predatory wasps were also observed feeding on honey-dew produced by aphids, which at times was produced in large enough quantities to make the *V. faba* leaves under the aphids look wet.

The wasps appeared to feed only on extrafloral nectar when other food sources were limited, such as when social wasp numbers peaked and prey grew scarcer late in the summer. The same two genera of predatory wasps (*Dolichovespula* and *Vespula*) were also found in Manitoba (Malaipan 1979), whereas only one vespid species of a different genus (Table 1.2) was recorded from a single visit in Massachusetts (Bugg *et al.* 1989).

Other than wasps, the only predatory insects observed consuming extrafloral nectar were coccinellid beetles (Table 2.1). Species from the family Coccinellidae were also reported on *V. faba* EFNs in other studies from both Canada and Europe (Hetschko 1908, Malaipan 1979), including two of the species found during the current study (Table 1.2). The other coccinellid observed at EFNs in this study was *Cryptolaemus montrouzieri*, a species endemic to Australia, but frequently used elsewhere for biological control. This beetle is thought to have recently escaped the University of Saskatchewan's greenhouses nearby, as the species is unable to survive winter in the local climate.

Previous studies have found that ants can reduce the number of other nectar consuming insects, such as parasitoid wasps (Cuautle and Rico-Gray 2003), which may have been a factor behind why a much larger number of other insects occurred at a plot with ants almost entirely absent (Crop Science plot) compared to the plots where ants were highly abundant (Biology Research plot). However, this does not explain the low number of visitors to the alternate patch in the nearly ant-free location, and neither parasitoid nor predatory wasps appeared to be visually deterred by the presence of ants when the wasps frequented other parts of the plants. No hostile encounters between ants and wasps were observed. One plot also received 20 visits to EFNs by various flies, most of which were lauxaniid flies. Although the flies were present in all plots in both years of the study, the particularly hot, dry, and windy weather that occurred while one plot was being surveyed was likely responsible for this difference in visitation numbers. Only one cultivar appeared to a have a substantially different number of non-ant EFN visitors, as Snowdrop only received a single visit during surveys of the plot that had recorded 29 non-ant visits to EFNs. In total, the EFNs of cultivar Snowdrop had only four out of the 54 visits by insects other than ants recorded throughout the study. Its lack of tannins does not appear to be responsible though, since the other tannin-free cultivar received a comparable number of visits to the tannin-rich cultivars.

Of the five ant species found at EFNs in this study, none appear to have been previously recorded on *V. faba* EFNs, though all five species are native to North America. However, other species in the genera *Lasius* and *Myrmica* are both known to consume *V. faba*'s extrafloral nectar (Hetschko 1908). All ant species present within the study's vicinity were found to have visited the EFNs of *V. faba*, in contrast to a study in Japan which found more ant species on *V. faba* that did not consume the extrafloral nectar, than species which did (Katayama and Suzuki 2003). The anti-herbivory effects of ants' presence were notable however, as the location containing many ant nests ended the first study season with substantially fewer aphids than the location without ants, where aphid numbers grew exponentially throughout the summer. As a result, other researchers at the Crop Science location sprayed insecticide throughout the entire area surrounding, but not including, the *V. faba* grown for this study, which is in part why statistical analyses of data for that location are not included here.

Although the same ant species were recorded both years of the study, significant differences in weather occurred between years. In 2014, slightly lower spring temperatures and much greater precipitation caused the soil moisture content to be unusually high (Fig. 2.6a,b). As a result, the first set of seeds planted in 2014 were lost due to mould.

2.4.1.1 Ants and extrafloral nectaries

The possible effects the presence or absence of tannins in *V. faba* could have on EFN visitors were explored for each ant species, which produced a variety of different results. For *Formica lasioides*, the cultivar did not appear to significantly influence which *V. faba* EFNs were fed from (Fig. 2.2a). Although fairly similar in appearance to *F. podzolica*, ants of *F. lasioides* were less easily disturbed, and could not move or escape capture as quickly as *F. podzolica*, which may have contributed to their much smaller numbers.

Like *F. lasioides*, the cultivar of the *V. faba* did not appear to be an important factor in determining which EFNs *F. neoclara* visited (Fig. 2.2b). However, the first plot of *V. faba* planted each year was responsible for 90% of the surveys with *F. neoclara* present at the EFNs. Rather than being due to a physical difference in location, as the plots were directly adjacent, the differences between plots were likely due to when the *V. faba* were planted in the respective plots. In addition to the initial absence of extrafloral nectar, differences in weed removal may have impaired *F. neoclara* colony growth in the plot seeded later in the year.

Although the plots were weeded equally often, weeding of the initially empty plot resulted in a lack of ground cover for a significant portion of the summer, preventing ant colonies started in the spring (or previous fall) from doing as well as those colonies in the plot seeded earlier. Colonies founded during the field season were unlikely to be visible until the next year, as alates/dealates of both *F. neoclara* and *F. podzolica* were only found in the area in early to mid-August. However, August to September is when other species' reproductive generations are also active locally, such as *L. pallitarsis* and *M. brevispinosa* (D. Wiens, pers. observ.), and ant colony reproduction in general is highly dependent on local weather compared to a precise time of year. *Formica neoclara* does not differ from the other ant species in that respect; therefore, the reason *F. neoclara* had a substantially reduced presence in the second plot compared to other abundant species may be due to the magnitude of the difference in how well the species survived in the presence compared to the absence of ground cover. Alternatively, the difference could just be due to species' variable survival rates in the face of the frequent disturbance caused by soil turnover, as there were fewer undisturbed patches of soil left after weeding occurred in the empty plot, compared to the plot which was already seeded.

In contrast to the other *Formica* species, the generalized mixed model for *F. podzolica* was improved by the inclusion of cultivar as an explanatory variable. Of all the ant species, *F. podzolica* was also the only species to have the cultivars with tannins group separately from those without tannins, although a cultivar with tannins still had some statistical overlap with a tannin-free cultivar (Fig. 2.2c). As well, if ant species happen to prefer two cultivars at random, one third of the species would be expected to choose either both tannin-free, or tannin containing cultivars. Once the other ant species preferences are taken into account, it seems likely the results for *F. podzolica* were less a result of a preference for tannin-free EFNs, and more probably just a result of the patches closest to the nests being composed of a slightly greater number of cultivars without tannins. Although the purpose of the random factors in the generalized mixed model were to account for such random differences, the variables may have in part been thwarted by the clumped distribution of ant visits, as the ants tended to forage close to their nests within the plot areas.

Like *F. podzolica*, the generalized mixed model for *Lasius pallitarsis* was improved when Cultivar was used as an explanatory variable. Only small statistically significant differences were found between cultivars (Fig. 2.2d). Despite those differences between

cultivars, the results did not suggest that tannin presence or absence was responsible, as cultivars of both kinds had more comparable ant visitation numbers to one another than the other cultivar of a similar tannin content. For *Myrmica brevispinosa*, all visits were to cultivars with tannins (Fig. 2.2e); however, the number of visits was not just too few, but also too clustered for statistical analyses between different cultivars. Throughout the study, no plot ever contained more than one nest of this ant species. Many of the different ant species' nests were also founded prior to the planting of *V. faba* seeds, so it is entirely possible that most, if not all of the apparent cultivar preferences, were a result of ants selecting the closest plants to their nests. Overall, the cultivar of plant used was not consistently useful as an explanatory factor when generalized mixed models were analyzed. Cultivars also had very similar numbers of species visiting during individual surveys.

In part, the reason why cultivars sometimes appeared to have significant differences in EFN visits by some ant species was probably because the random factors did not account for variance as clearly or effectively for ants as they would have for foraging insects with wings, although all random factors accounted for at least some variance (Table 2.2). Ants were consistently found in the patches of plants where their nests were located, and relatively frequently fed from EFNs of *V. faba* in patches adjacent to the nests, resulting in a clumped, non-random distribution of patches with a particular ant species present at the EFNs. On a larger scale, the random distribution of nests would have likely been captured by the use of random factors; however, the experiment was originally designed to account for the type of variability caused by transitory visitors, which would have been at the scale of individual patches or blocks of plants, rather than the clumps of patches visited by entire colonies of ants.

When ants did appear further outside of the area where there nests were based, the ants appeared to show scouting behaviours, especially in plots already occupied by the nest of another ant species. Extrafloral nectar consumption was infrequent for these rapidly moving ants and did not last nearly as long as foraging workers, which would often consume extrafloral nectar from several consecutive nectaries on a plant. Hostility between ant species, although only occasionally observed, did occur. In one such incidence, four *F. neoclara* workers dragged a still living *L. pallitarsis* into their nest, presumably for future consumption. However, more often, already deceased ants were carried into nests during foraging activities.

Differences in aggression, as well as other natural history characteristics, may have also been responsible for the variation between ant species in regards to the number of surveys with a particular species present at the EFNs. While *F. lasioides* and *M. brevispinosa* had at most one colony per plot each year, species such as *F. podzolica*, *F. neoclara*, and *L. pallitarsis* had numerous colonies in each plot, for each year that the surveys took place in the Biology Research gardens area. The difference in colony number may have been due to differences in reproduction, movement speed, and predation-avoidance strategies, as those natural history characteristics appeared much more variable between species than their use of *V. faba* EFNs, which were fed on extensively by all ant species.

Although examining differences in natural history for the ant species present was not a primary goal of the study, several traits and behaviours which may have influenced the number of colonies were noted. *Formica podzolica* was recorded at EFNs from the largest number of surveys (83/144), and was also distinctly more difficult to catch than other ant species. Although it was only somewhat faster than other *Formica* species, *F. podzolica* was alone in its ability to frequently escape capture attempts by simply walking off the leaf it was on, and dropping either straight to the ground, or to a lower part of the plant. Other species generally tried to make their way down the stem, while staying on the side of the stem or leaf that was not visible to their pursuer. *Formica podzolica* was also the most attentive to shadows or plant movement induced by the researcher, and presumably would have escaped the most easily from other predators as well. In contrast, *L. pallitarsis* and *M. brevispinosa* moved much more slowly than *Formica* species. Although *L. pallitarsis* was still relatively common throughout the surveys (19/144), this species frequently tolerated other ant species on *V. faba* EFNs in the vicinity of their nests, possibly due to its smaller size. Defensive behaviours also differed between species, as *M. brevispinosa* is able to sting, whereas the four formicine species can spray formic acid at predators in an attempt to amplify the effect of potential wounds caused by their bites, or make the ants distasteful. Additionally, when the acid was not employed, flavours seemed to vary significantly between species. In general, distinctions between ant species' behaviours and physical traits appear to be likely reasons for the variability in the ants' visits to different cultivars.

2.4.1.2 Extrafloral nectary relative position

The ant species *F. neoclara* and *F. podzolica* also differed slightly, with either each other and/or with the other insect species, in regards to the relative locations of the EFNs they visited on *V. faba* (Fig. 2.3a-c). However, although the difference was statistically significant, the very small effect size indicates that it likely has no biological significance, as the visited node's average relative position for the different species/categories varied by, at most, 0.5 nodes from one another. The statistical difference could be due to slight physical differences in the plants of individual patches or areas in the plot, as the ant species tended to have a clumped distribution throughout the plot. However, the slight differences between insect species categories could also be a result of the varying sizes of each category, which was not the case for the observations regarding the different cultivars. The Chi-squared test comparing the relative position of the visited EFNs to *V. faba* cultivar indicated that cultivar was independent of which EFN was visited. The results suggest tannins therefore did not have an effect on which EFN location was visited on *V. faba.*

When analyzed separately, insect species categories (Fig. 2.3a-c) and *V. faba* cultivars (Fig. 2.4a-d) typically had the Primary Active EFN (Node 0) visited most frequently, with the average distance in nodes from the Primary Active EFN ranging between -0.1 and -0.5, indicating that the nodes basal to the Primary Active EFN were visited slightly more frequently than those apical to it. Despite the visited EFN relative location trends, the cluster of more exposed EFNs near the top of the plant appeared to offer an equal amount of nectar over a much shorter distance than the EFNs below the Primary Active EFNs. However, the more basal EFNs would have been encountered on the way to and from the Primary Active EFNs, making all the EFNs near the Primary Active EFN worth visiting for flightless, nectar-seeking insects. On average, the surveyed *V. faba* had more nodes located basal to the Primary Active EFN than apical to it, which the insect visit trends also appear to reflect. Additionally, ants were distinctly more visible when visiting EFNs apical to the Primary Active EFNs, but this did not appear to significantly deter them. Instead, the frequency of visits by ants may have caused the most productive of the EFNs to be relatively depleted throughout the day, which would have encouraged the ants to seek the nectar of other functional, although less productive, EFNs. With almost all the visits to EFNs occurring near the Primary Active EFNs, it appears that the stipular EFNs very likely peak in nectar production at the position of being the pair of Primary Active

EFNs, then gradually decline. Extrafloral nectar was only rarely consumed considerably further from the most active region, and at some point, nectar production did stop for EFNs, as EFNs near the base of maturing plants were visibly dry and were no longer visited by any insects.

Occasionally, insects would also consume nectar from an EFN located on a leaf tendril, one of which often extended from the end of *V. faba* compound leaves (Fig. 2.7a,b). Visits to tendril EFNs occurred for all four cultivars used in the study, and visits to them by both *F. neoclara* and *F. podzolica* were recorded. The relative positions of the visited leaf tendril EFNs were similar to that of stipular EFNs, with the most visits occurring at a tendril extending from the same node as the Primary Active EFNs, and the majority of visits occurring no more than one node away. Further refining of the definition for the Primary Active EFN location might allow a single relative location on the plant to account for a greater proportion of the insect visits; however, using either relative, absolute, or a combination of the two types of measurements to define the most productive EFNs location may be problematic. Variability in the plants' morphology place constraints in what otherwise may intuitively be thought of as the first set of stipules below the apical cluster of stipules, as *V. faba* vary in stipule size and internodal length between various cultivars, seedlings and mature plants, as well as between plants grown outside compared to those in a greenhouse.

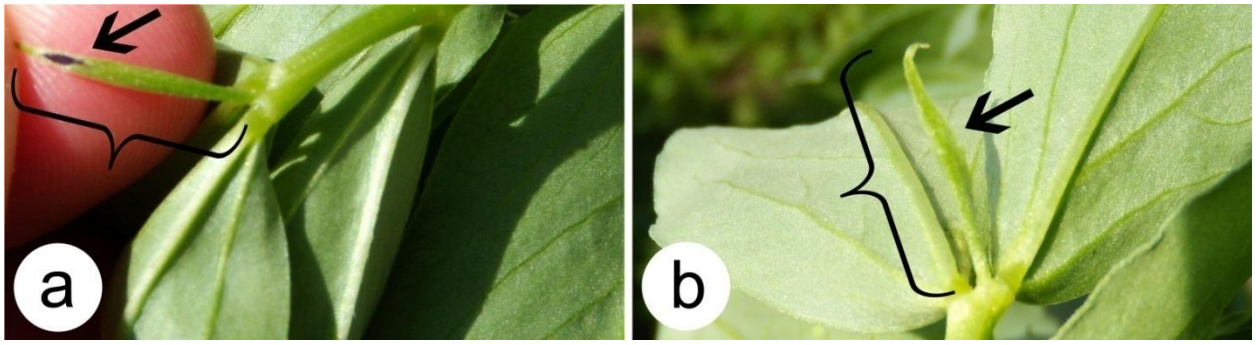


Figure 2.7 Extrafloral nectaries on leaf tendrils of *Vicia faba* that contain tannins (a) and lack tannins (b). Brackets indicate the location of tendrils, and arrows point to the location of EFNs.

2.4.2 Factors affecting insect visitation to floral nectaries

The majority of floral visitors found during 2013 surveys were species of *Bombus,* which also made up a sizable portion of the visitors from 2014 (Fig. 2.5a,b). In 2014, all the insects visiting *V. faba* during surveys were of the bee family Apidae, most of which were *Apis mellifera. Bombus* species and *A. mellifera* were common in a number of past studies as well

(Kendall and Smith 1975, Kołtowski 1996c, Malaipan 1979, Poulsen 1973), and where they were not, other species of Apidae usually were (Aouar-sadli *et al.* 2008, Benachour *et al.* 2007). Regardless of geographical location, the majority of visitors to *V. faba* flowers generally appear to be members of Apidae. However, *V. faba* has been grown for much longer and in greater quantities in European and North African countries, and past research has been correspondingly less in North America. As a result, *Bombus nevadensis* does not appear to have been previously recorded as a visitor to *V. faba*, despite *B. nevadensis* being the native bee most frequently found at *V. faba*'s flowers during this study. Previously recorded visits to *V. faba* flowers were also not found for *B. huntii* and *B. perplexus*, though neither appeared to visit the flowers in significant numbers.

Although flower visitation rates were low in the present study, there were a number of highly abundant pollinators in the area that were found to have only visited flowers on one or two occasions, if at all. *Apis mellifera* was absent from the Biology Research plot surveys in 2013, however it was present in low numbers at other plants nearby. Flower-visiting species such as *B. huntii, Halictus rubicundus*, and various syrphid species were all present in the area in large numbers, but were more likely to be found resting on leaves of *V. faba* than attempting to consume floral nectar, or gather pollen. However, solitary bees, wasps, and syrphids, including those in Table 2.1, lack the required tongue length to consume floral nectar, and therefore likely avoided *V. faba* after a few unsuccessful feeding attempts. Bees and other potential pollinators did not appear to be deterred by the presence of ants though, and only a single instance of hostility between an ant and bee was observed. In that particular instance, the actions of the *B. ternarius* worker were quite irregular, as it remained on a leaf of *V. faba* for a prolonged period of time, crawling in circles repeatedly. As such, no bees were seen repelled from flowers by ants, and ants were generally not present on the racemes in any case.

General linear models of the flowers' most commonly visiting insect species did not suggest *V. faba*'s cultivar contributed enough to the models to support its inclusion. Instead, the null models had the best fit relative to their complexity (Table A.1). Despite the low number of visitors to flowers of all cultivars, there did not appear to be substantial differences in the number of visits between cultivars, which is in agreement with past work (Malaipan 1979). However, species richness did vary between cultivars, with the cultivar SSNS-1 having twice the number of visiting species compared to Snowdrop. The variation was of only three species though, so it

may have been reduced substantially with a greater sample size, and the remaining two cultivars were intermediate with four species of insect visiting each. Species abundance showed the same patterns as richness, and also did not suggest any associations with tannin content. The presence or absence of tannins therefore did not appear to have any noticeable effect on insect visitors to the flowers of *V. faba* in this study.

In contrast to previous studies (Free 1962, Kołtowski 1996c, Malaipan 1979, Poulsen 1973), there did not appear to be any instances of nectar-robbing during surveys, and no flowers with holes at the base of the corolla were observed. The lack of nectar thieves came in spite of the abundance of *B. terricola* and *A. mellifera* in the area, which are both known to steal nectar through holes at the flowers' base. However, *A. mellifera* generally only steals nectar through pre-existing holes, such as those made by *Bombus* (Free 1962). Few bees which actually bite holes in the flowers visited during the study though, as *B. terricola* was far more abundant on nearby flowers than on those of *V. faba*. This was likely due to the relatively small number of *V. faba* (less than 1000 plants) within the present study's areas, as many other floral resources were easily available in the immediate surroundings. The previously mentioned studies were conducted in fields of one or more hectares of *V. faba*, which would have placed much greater constraints on locally nesting bees searching for accessible food sources. As a result, shorter-tongued *Bombus* may have been more likely to create the holes necessary for them to easily access a nearby source of floral nectar, as the constituent sugars of extrafloral nectar do not necessarily have the same appeal to bees as the sugars found in floral nectar.

3 Extrafloral nectar production in *Vicia faba*

3.1 Introduction

In addition to varying in location, structure, and appearance, the extrafloral and floral nectaries also differ in the quantity and composition of the nectar they produce. The EFNs have been found to produce a volume of nectar around 10% greater per stipule than floral nectaries per flower; however the nectar contains 43% less sugars per stipule than is found per flower (Davis *et al.* 1988). The lower concentration of the sugars in the extrafloral nectar is possibly reflective of the high proportion of xylem tracheary elements beneath each stipular nectary. Nectar sugar concentrations were measured as 44.7% in floral nectar, and 25% in extrafloral nectar of the Outlook cultivar (Davis *et al.* 1988). In the Polish cultivars Dino, Grot, Gryf, Nadwiślański, Stego, and Tibo, nectar sugar concentrations were 20-60% (averaging between 30-40%) for floral nectar, and were 40-60% for extrafloral nectar, with an average sugar mass of 9 mg for 10 flowers, and 0.8 mg for 10 extrafloral nectaries (Kołtowski 1996b). No significant differences among nectar characteristics were found between 5 of the 6 Polish cultivars, whereas cultivar Tibo produced less extrafloral nectar sugar.

Although past studies have briefly examined extrafloral and floral nectar composition, the results are highly divergent, and have been produced using very different methods. Using semi-quantitative, thin-layer chromatography, extrafloral nectar was found to contain only trace amounts of sucrose, and was composed of mainly glucose and fructose, whereas only sucrose was detected in floral nectar (Davis *et al.* 1988). In contrast, high performance liquid chromatography (HPLC) using entire stipules which were first frozen in liquid nitrogen, then ground up, produced measurements of 0.2 ± 0.0 (mM/10 mg dry weight of plant tissue) fructose, 1.5 ± 0.2 glucose, 0.3 ± 0.1 inositol, and 0.9 ± 0.1 sucrose (Irvin *et al.* 2007). Despite the greater accuracy of HPLC compared to thin-layer chromatography, the sugar content of entire stipules is only crudely representative of the stipules' of extrafloral nectar, as invertase activity can alter nectar chemistry, post-secretion (Heil *et al.* 2005b), for example.

Invertase activity results in equal amounts of glucose and fructose; however, the HPLC analysis of stipular tissues found the amount of glucose was several times greater than the amount of fructose (Irvin *et al.* 2007). Although extrafloral nectar chemistry is less well studied than that of floral nectar, variable glucose:fructose ratios are a common occurrence in floral nectars, with one study finding a 1:1 ratio in only four species of the 137 nectar producing plant

species examined (Baker *et al.* 1998). Several explanations have been proposed for why nectar glucose:fructose ratios may differ from 1:1, including microbial activity (Lüttge 1961) and the presence of oligosaccharides (Lüttge 1962). Although the microbial content of *Vicia faba* extrafloral nectar is not currently known, microbes from other nectars are known to create glucose:fructose ratios which favour fructose, rather than glucose (De Vega and Herrera 2013). Nectars are known to sometimes contain oligosaccharides as well (Baker and Baker 1983), which can be formed from sucrose through enzymatic processes resulting in unequal amounts of glucose and fructose (Wolf and Ewart 1955). Uneven glucose:fructose ratios can also result from some of the sugars going through part of, or the complete cycle of glycolysis, gluconeogenesis, and the pentose phosphate pathway (Wenzler *et al.* 2008). Additionally, nectar resorption can sometimes alter nectar sugar ratios (Nepi *et al.* 2001, Nicolson 1995). Although the effect of nectar resorption on nectar sugar composition is currently unknown (Nepi and Stpiczyńska 2008), there is evidence that nectar resorption occurs in EFNs. Recently, nectar resorption has been demonstrated in the EFNs of *V. faba* and *Gossypium hirsutum* (Cardoso-Gustavson and Davis 2015), and previous evidence suggests it may occur in other plants as well (Mohan and Inamdar 1986).

Several new cultivars of *V. faba* are being selected for their absence of tannins, which can interfere with the digestibility of their protein content (Crépon *et al.* 2010). Noteworthy phenotypic features among tannin-rich cultivars include the presence of black patches on the wing petals and dark-pigmented EFNs on the stipules (Fig. 1.1a,c), whereas tannin-free cultivars lack this pigmentation (Fig. 1.1b,d). The lack of colouration in the flowers and EFNs in tannin-free cultivars is attributable to a lack of anthocyanins (Cardoso-Gustavson and Davis 2015), which may indicate a complete deficiency in the flavonoid pathway. Tannins are often used by plants to reduce herbivory, the lack of which may negatively affect the plants (Coley 1986, War *et al*. 2012). Differences in genotype are also known to significantly influence extrafloral nectar production in other plants, such as *Populus tremuloides* Michaux (Newman *et al.* 2016). To explore whether differences in insect visitation to *V. faba* cultivars could be due to factors other than EFN colour, the objectives of this study were to compare the volume, concentration, total nectar sugar, and carbohydrate composition of the extrafloral nectar of four cultivars, two with tannins present and two without. The hypothesis tested is that these characteristics of the

extrafloral nectar produced by stipules of *V. faba* may vary with the tannin content of the cultivars investigated.

3.2 Materials and Methods

3.2.1 Plant growth protocol

Plants of *V. faba* were grown in an indoor facility with controlled environmental conditions, in order to provide a suitably humid environment for nectar sampling. The same four cultivars (Fatima, SSNS-1, Snowbird, and Snowdrop) used in the field study (Chapter 2) were investigated for their nectar characteristics. Plants were grown using the following protocol: seeds were planted 2.5 cm deep in potting soil (Sunshine #1 mix, Seba Beach, AB) inside separate plastic pots, which were 10 cm at their greatest diameter. The growth chamber was kept at 25°C from 5:00 am to 8:00 pm while the fluorescent and incandescent bulbs were lit, and 19°C during the night when the growth chamber was left dark. Plants were watered two to three times a week with 100-150 mL per pot, with the water delivered directly to the potting soil via a beaker, to prevent the water from contacting the extrafloral nectar. Before each watering, plants were relocated throughout the growth chamber to ensure that plants of all cultivars received equal amounts of light.

3.2.2 Nectar sampling

Extrafloral nectar was sampled one day after watering, at every second node of the pre-flowering plants with active EFNs, for a continuous period until the EFNs were no longer functional. Nodes were sampled soon after they appeared, starting with the third node of each plant, because the first node did not appear to have a stipular EFN, and was often located level with the soil. The EFNs of the second node were not consistently functional either, and neither of the first two nodes had leaves arising from them, unlike all nodes which grew after them. The plants were sampled during growth stage 1 (leaf development, BBCH codes 11-14) according to the BBCH growth stage scale for faba beans (Lancashire *et al.* 1991, Weber and Bleiholder 1990). In the first trial, 45 plants of the Snowdrop cultivar alone were grown and sampled for extrafloral nectar at 2 hr intervals from 5:00am to 5:00pm. Four different plants were sampled at one node for each time point throughout the day, and no additional samples were taken from the same plant again until approximately a week later when the next sampling date occurred.

Following the preliminary trial with the Snowdrop cultivar, three additional trials were conducted, with all sampling for extrafloral nectar taking place during its peak appearance (ie., 10:00 am to 12:00 pm). Each trial consisted of 20 to 30 plants of each of the four *V. faba* cultivars. Between trials, the growth chamber was emptied of plants, heated to 30°C for several days, then left to cool off, in an effort to desiccate insect pests (thrips) in the chamber. Non-chemical pest control measures were also used during two of the trials, including yellow sticky traps in one trial, and predatory mites (*Amblyseius swirskii* Athias-Henriot). EFNs partially damaged or rendered entirely non-functional by thrips were not sampled, and could be distinguished from undamaged EFNs which failed to produced nectar.

3.2.3 Nectar refractometer measurements

Individual samples of extrafloral nectar were taken for concentration and volume measurements by microcapillary collection of the nectar using 1 µL Drummond Microcaps®. At each nectar sampling, a separate Microcap® was used to collect all nectar available per EFN for each pair of stipules per node. Nectar volume was measured using a dissecting microscope (Olympus SZ40) and by placement of a ruler with 1 mm markings beside the partially filled Microcap®. Nectar sugar concentration by weight (NCW), expressed in g sucrose (or sucrose equivalent) / 100 g solution, was measured using hand-held nectar refractometers (0 - 50, 40 - 85%; Bellingham and Stanley, Tunbridge Wells, U.K.). Nectar concentration by weight was converted to nectar concentration by volume (NCV), measured as g sugar/mL solution, using the following quadratic equation from Búrquez and Corbet (1991): $NCV = NCW^2 (59.6 \times 10^{-6}) + NCW (9.224 \times 10^{-3}) + 7.08 \times 10^{-3}$. For nectar with 10 - 80% solute concentration, the nectar concentration conversion equation results in error values of less than 1% (Búrquez and Corbet 1991). These NCV values were used in conjunction with volume measurements to determine the mass of sugar from the nectar of both stipules belonging to one node of a plant. Nectar sugar masses were then divided by two to give the average mass of nectar sugar per stipule.

Eight plants of each of the four cultivars had their nectar solute concentrations assessed by refractometry, twice for each of two replications (n = 128). Samples were taken starting at 11:00 am. Occasionally, sugar-rich nectar samples were used up (n = 11) by the refractometer which only measured concentrations up to 50%, and therefore the precise sugar concentration could not be determined. For the samples with unknown concentrations at or above 50%, nectar

concentration values were replaced with the average of all concentrations measured accurately by the other refractometer to be 50% or greater.

3.2.4 High Performance Liquid Chromatography

For HPLC testing of carbohydrate composition of *V. faba* extrafloral nectar, four plants per cultivar were sampled at each of two nodes for two trials (n = 64). Samples were taken using small filter-paper wicks that were dried and stored prior to compositional analyses (McKenna and Thomson 1988). Generally, all nectar available from both stipules of a sampled node was taken up on one wick, although a few samples were attempted from single stipules as well. Most samples taken from single stipules were later combined prior to HPLC analysis, in order to ensure sufficient sugar per sample to be detectable, and enough eluent to have duplicate injections run. The extrafloral nectar samples on paper wicks were dissolved into 500 µL double distilled water (250 µL per stipule sampled), syringe filtered (0.2 µm pore size), and run through a Dionex ICS-5000 HPLC. The isocratic flow rate was 1.0 mL/minute using an 80 mM NaOH mobile phase. A Dionex Carbo-Pac PA1 analytical column (4 x 250 mm) was used for carbohydrate separation, run in series after a guard (4 x 50 mm) of the same stationary phase. For the 50 samples that ran successfully, the resulting chromatographs were analysed using Chromeleon 7 software. Standard curves were prepared for fructose, glucose, and sucrose in 10-100 mg/L solutions using double distilled water. Duplicate injections of both samples and standards were run, and all standard curves were linear with R^2 values of ≥ 0.997.

3.2.5 Statistical methods

Data collected from refractometer readings and HPLC was analyzed using general linear models. Potential explanatory factors in maximal models were tested for interactions and their effect on the model; those that did not contribute to the models' goodness of fit were eliminated to increase the degrees of freedom as long as the new model would have an AIC (Akaike's Information Criterion) value lower than, or within two points of, the previous model. The maximal models included which cultivar and node was sampled, the greenhouse trial the plants were a part of, and the interactions between all the potential factors.

Minimally adequate models had more degrees of freedom, and significantly (>2) lower AIC values than maximal models, indicating that the selected models fit better. F-tests were conducted to ensure the maximal and minimal models' explanatory power was not significantly

different for each set of models. The minimal models were also tested against null models using F-tests to ensure they were significantly different in explanatory power, and checked for a drop in AIC values as well. Orthogonal contrasts with single coefficients were used in the post-hoc analyses of potential node and cultivar differences, with the Holm-Bonferroni correction method applied to the resulting *p*-values (Holm, 1979). Further model information, such as the AIC values for the models examined during the selection process, is detailed in Appendix 1.

3.3 Results

3.3.1 Extrafloral nectar sugar mass per stipule

Using a combination of microcapillary collection followed by refractometer measurements from two of the trials (n = 128), the average mass of nectar sugars per stipule was calculated for each measurement, and then modelled. One trial was not included in the data, as thrips rendered the majority of EFNs non-functional prior to sufficient nectar sampling taking place. The null model was rejected and the selected general linear model retained both cultivar and node as significant factors (Table A.1). Additionally, there was a significant interaction between cultivar and node (Table A.3). Cultivars did not group by tannin presence or absence when the average mass of sugar produced per stipule was compared between cultivars, although there were other significant differences between various cultivars (Fig. 3.1, Table A.2). Post-hoc testing using orthogonal contrasts revealed consistently larger sugar quantities for measurements taken at the fifth node, compared to the third node of *V. faba* (Fig. 3.1). The overall average mass ± S.E.M. for the extrafloral nectar sugar per stipule was 137.6 ± 10.0 µg. The average sugar mass ± S.E.M. for Fatima, SSNS-1, Snowbird, and Snowdrop extrafloral nectar was 158.6 ± 19.8 µg, 66.8 ± 14.2 µg, 187.6 ± 16.3 µg, and 137.5 ± 22.0 µg, respectively. Nectar taken from the third-node EFN of the above cultivars had an average of 86.9 ± 7.5 µg of sugars, whereas the average for the fifth node was 188.9 ± 16.2 µg.

To check if samples used for refractometer and HPLC measurements produced similar results for the amount of nectar sugar produced per stipule, the mass of extrafloral nectar sugars from HPLC samples was also calculated and modelled (Table A.4). The resulting model significantly improved upon the null model (Table A.1). With this smaller data set (n = 50), the average nectar sugar mass wicked per stipule was 147.6 ± 15.5 µg, which was not significantly

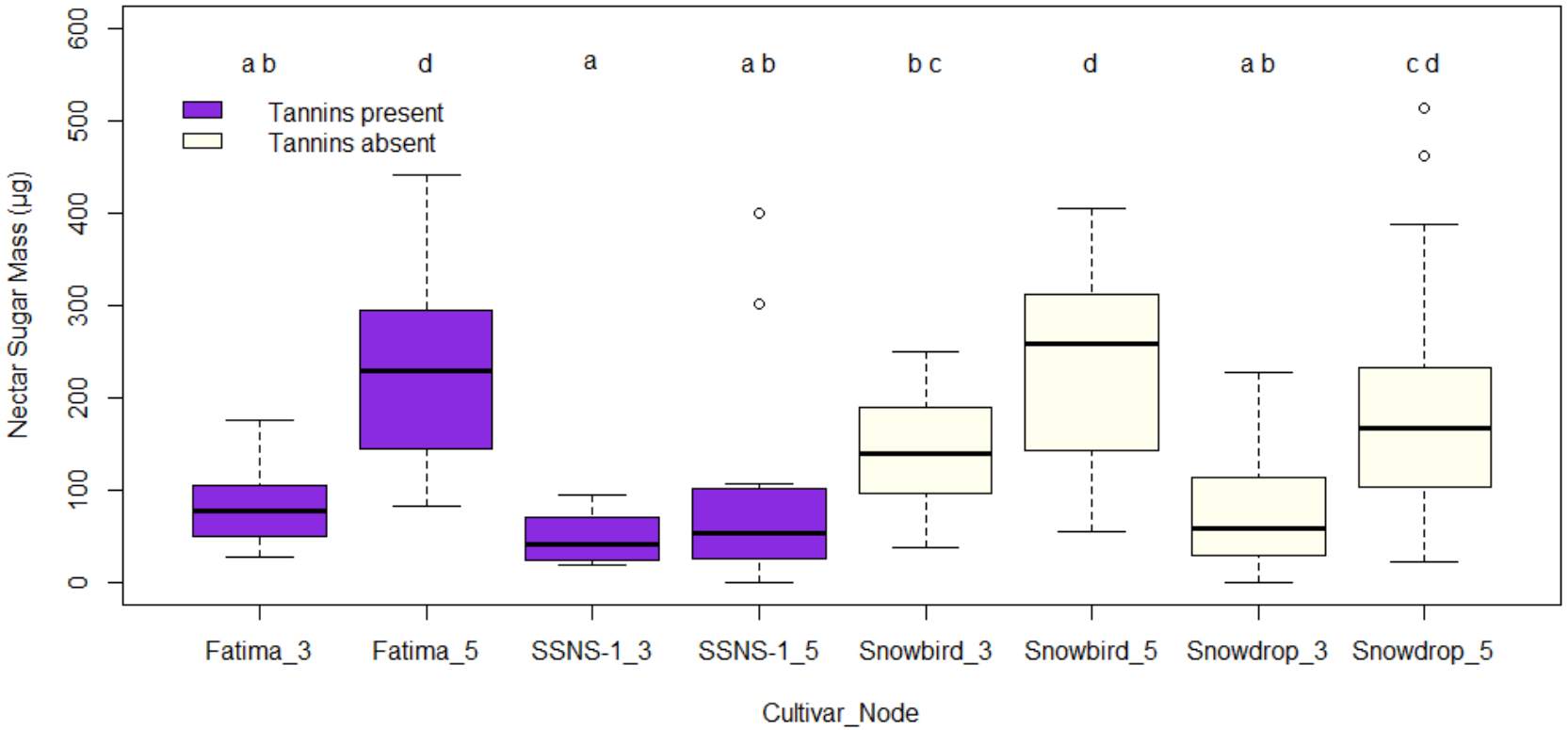


Figure 3.1 Nectar sugar mass (µg) of *Vicia faba*'s extrafloral nectar per stipule, categorized by both cultivar and node. Each recorded nectar sugar mass represented the average of the two extrafloral nectaries found at the node sampled. Eight plants from each of two experiments were measured, for a total of 16 plants for each node and cultivar combination (n = 128). For each cultivar and node combination, the median 50% of data is contained in a box composed of lines for the 25th and 75th percentile, as well as a thicker dark line for the median. Whiskers represent the upper and lower 25 percentiles, except for the outliers (circles), which are at least 1.5 times greater than the 75th percentile.

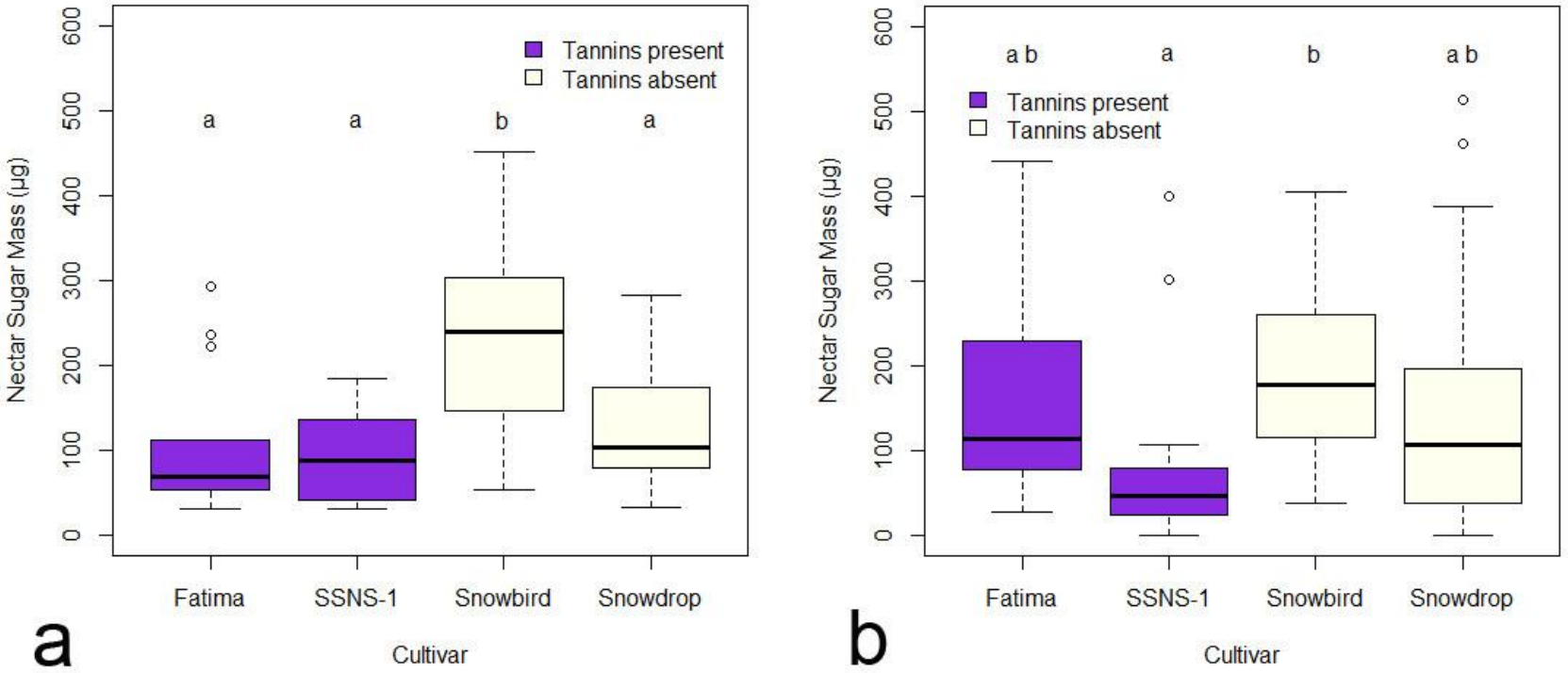


Figure 3.2 Nectar sugar mass (µg) of *Vicia faba*'s extrafloral nectar per stipule for each of four cultivars. Each recorded nectar sugar mass represented the average of the two extrafloral nectaries found at the node sampled. Nectar sugar mass was calculated using a) HLPC analysis of wicked nectar (n = 13, 12, 14, and 11 for Fatima, SSNS-1, Snowbird, and Snowdrop, respectively), and b) a combination of microcapillary and nectar refractometer readings (n = 32 per cultivar).

different than the overall average collected by microcapillary and then calculated following refractometer measurements (Welch's t-test, *p*-value = 0.59). Using the HPLC data, Snowbird was the only cultivar to be statistically different from the other cultivars, and SSNS-1 had, on average, the smallest amount of sugar produced per stipule (Fig. 3.2a, Table A.2). Similarly, data derived from refractometer measurements showed Snowbird and SSNS-1 were the only cultivars to be significantly different from one another, as they were once again the cultivars which produced the greatest, and least amount of extrafloral nectar sugar, respectively (Fig. 3.2b, Table A.2).

3.3.2 Extrafloral nectar volume and concentration

Overall, the average extrafloral nectar volume per stipule ± S.E.M. was 0.363 ± 0.021 µL, and the average extrafloral nectar NCW per stipule was 32.5 ± 1.3 %. The same procedure as was used for nectar sugar mass was also used for comparing nectar volume and concentration between cultivars. For nectar volume, the selected general linear model kept cultivar, node, and the interaction between them as statistically significant factors (Table A.5). Orthogonal contrasts used in post-hoc testing showed statistical differences for various cultivar/node combinations; however, cultivars did not group by tannin presence or absence (Fig. 3.3, Table A.2). The average volume of nectar per stipule ± S.E.M. for cultivars Fatima, SSNS-1, Snowbird, and Snowdrop extrafloral nectar was 0.433 ± 0.039 µL, 0.188 ± 0.037 µL, 0.456 ± 0.031 µL, and 0.373 ± 0.043 µL, respectively. Nectar volume from EFNs of the third node of *V. faba* cultivars averaged 0.318 ± 0.025 µL, and the average for the fifth node's nectar was 0.407 ± 0.033 µL. Minimally adequate models for both volume and concentration were compared to their respective null models with F-tests and AIC values; both F-tests resulted in significant *p*-values, and significantly lower AIC values in minimally adequate models than in null models (Table A.1).

Similarly, a general linear model was selected for evaluating NCW. The model included cultivar, node, the interaction between the former and latter, as well as trial, as those factors had a significant effect on the model (Table A.6). Post-hoc tests were performed with orthogonal contrasts, which did show statistical differences between many of the cultivar and node combinations; however, the cultivars did not group by tannin presence or absence (Fig. 3.4,

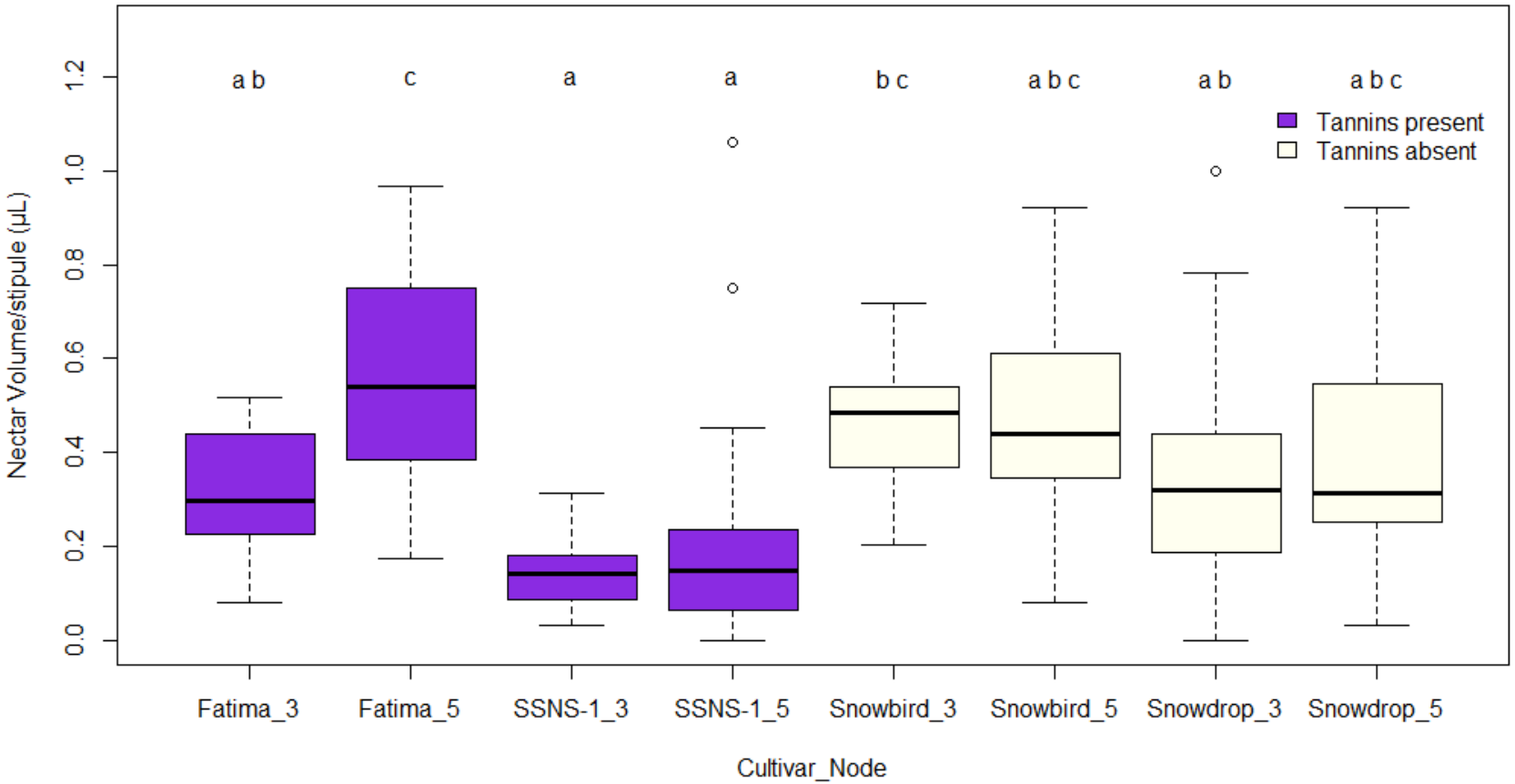


Figure 3.3 Volume of extrafloral nectar per stipule (µL) from *Vicia faba*, categorized by both cultivar and node. Each recorded volume represented the average of the two extrafloral nectaries found at the node sampled. Eight plants from each of two experiments were measured, for a total of 16 plants for each node and cultivar combination (n = 128).

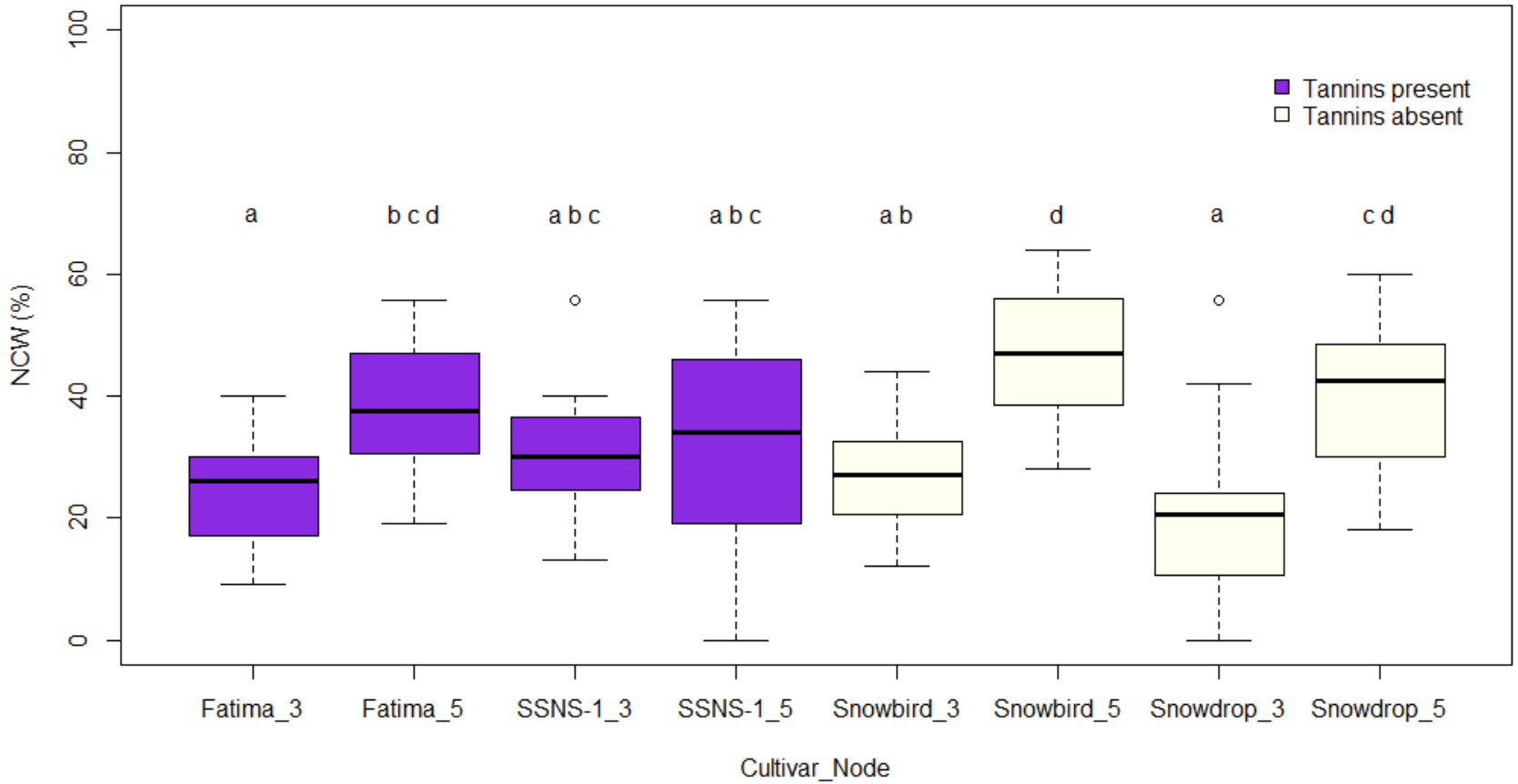


Figure 3.4 Nectar sugar concentration (%) by weight (NCW) of extrafloral nectar from *Vicia faba*'s stipules, categorized by both cultivar and node. Each recorded NCW represented the average of the two extrafloral nectaries found at the node sampled. Eight plants from each of two experiments were measured, for a total of 16 plants for each node and cultivar combination (n = 128).

Table A.2). Node 5 had a significantly greater NCW than Node 3 for every cultivar except SSNS-1. For cultivars Fatima, SSNS-1, Snowbird, and Snowdrop, the average NCW was 31.4 ± 2.0%, 31.2 ± 2.7%, 36.7 ± 2.4%, and 30.9 ± 2.9%, respectively. The average NCW for the third node of the cultivars was 26.1 ± 1.4% and 39.0 ± 1.8% for the fifth node.

3.3.3 Extrafloral nectar composition

When examined as a whole, extrafloral nectar of *V. faba* sampled in this study had an average nectar sugar composition of 54.4 ± 1.0% glucose, 31.1 ± 1.0% fructose, and 14.5 ± 1.0% sucrose. Occasionally, trace amounts of polyols were detected as well. For each of the nectar sugars, selection for the minimally adequate model resulted in the replication of the experiment to be dropped as an explanatory factor, and in all cases cultivar was kept as a significant explanatory factor (Tables A.7-A.9). For glucose, however, the node sampled also was kept as an explanatory factor, although the interaction between the nodes' position and the plants' cultivar was not statistically significant (Table A.7). F-tests comparing the minimally adequate models with the null models for each of fructose, glucose, and sucrose, resulted in significant *p*-values (Table A.1). In addition, all three minimally adequate models had a significantly lower AIC value than their respective null models.

Orthogonal contrasts, each using only a single coefficient, determined which cultivars had nectar sugar proportions which were statistically different (Table A.2). Figure 3.5a shows the proportion of sucrose in nectar of Fatima was not statistically different from that of SSNS-1 or Snowdrop, whereas Snowbird had a proportion of sucrose in the extrafloral nectar that was statistically different from all other cultivars (Fig. 3.5a, Table A.8). HPLC analysis of the sugar composition of *V. faba* extrafloral nectar revealed relatively low concentrations of sucrose, with the percent of nectar sugar comprised of sucrose (by weight) varying from 2.6 to 35.5%, with an average of 14.5% across all cultivars. For Fatima, SSNS-1, Snowbird, and Snowdrop, the average percent sucrose in extrafloral nectar was 13.3 ± 1.6%, 14.7 ± 1.5%, 20.3 ± 2.1%, and 8.5 ± 1.2%, respectively.

The overall proportion of fructose in extrafloral nectar was also fairly variable, ranging from 13.5% to 44.6% of the total nectar sugars. For cultivars Fatima, SSNS-1, Snowbird, and Snowdrop, the average percent fructose was 35.0 ± 2.0%, 27.8 ± 2.1%, 28.9 ± 1.4%, and 33.1 ± 1.6%, respectively. Post-hoc tests showed a statistical difference only between cultivars Fatima

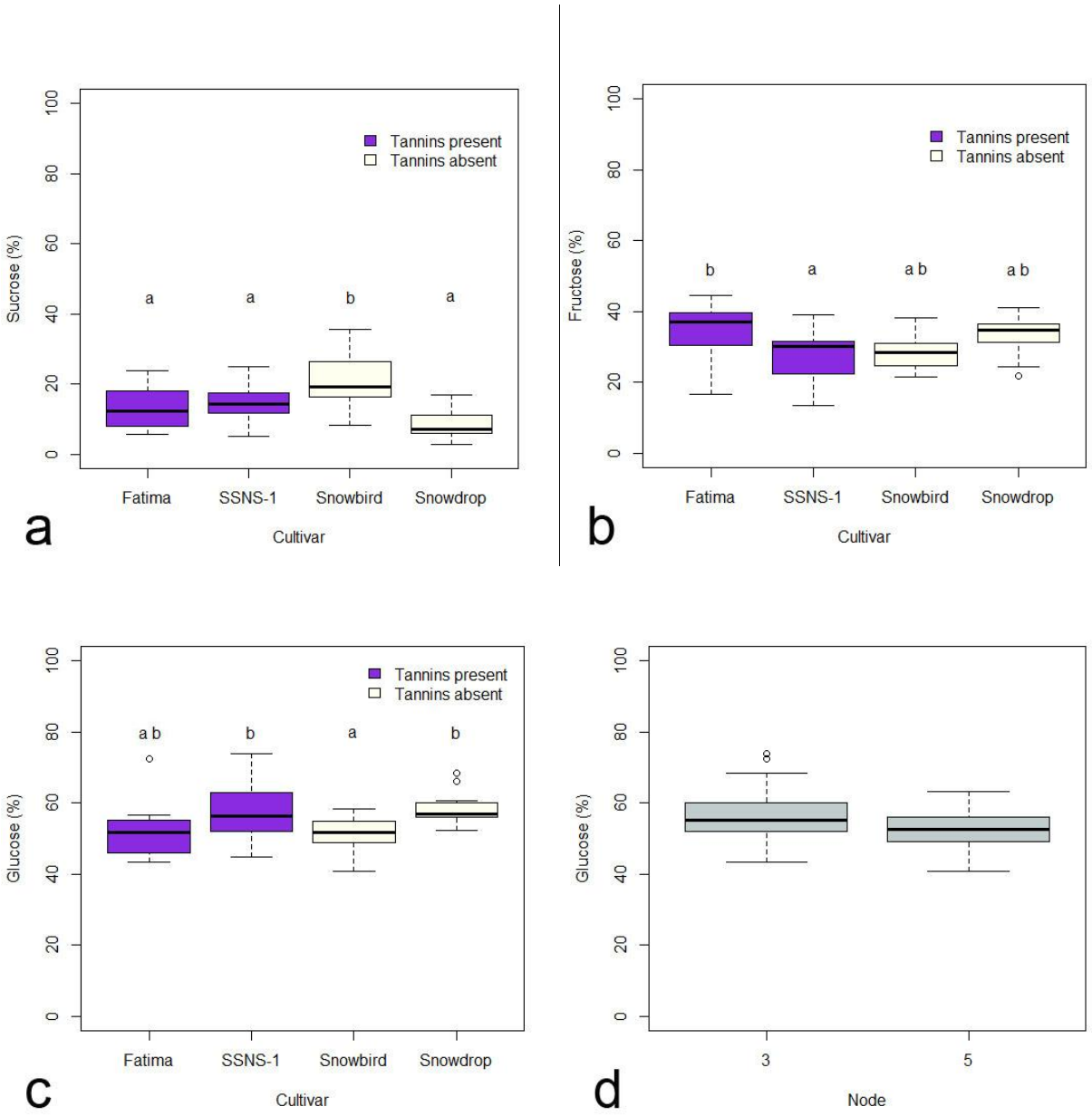


Figure 3.5 Proportion (%) of three types of carbohydrates analysed in *Vicia faba*'s extrafloral nectar by high performance liquid chromatography. Each recorded proportion (n = 128) represented the average for nectar drops wicked at the two extrafloral nectaries found at the node sampled, and were grouped by cultivar (a,b,c) or node (d). Analyzed nectar sugars were sucrose (a), fructose (b), and glucose (c, d).

and SSNS-1, with no overall difference found between those cultivars with tannins compared to those without (Fig. 3.5b, Table A.2).

Orthogonal contrasts also revealed a statistical difference between glucose proportions in the extrafloral nectar of individual cultivars (Fig. 3.5c). When grouped together, cultivars with tannins present compared to those with tannins absent revealed no apparent differences regarding the proportion of glucose contained in their extrafloral nectar. The average proportion of glucose for cultivars Fatima, SSNS-1, Snowbird, and Snowdrop was 51.8 ± 2.1%, 57.5 ± 2.3%, 50.8 ± 1.3%, and 58.5 ± 1.4%, respectively. The range for the relative amount of glucose in one stipule's nectar drop varied from 40.8% to 73.9% of the total sugar content.

The two nodes sampled from young plants of *V. faba* had very similar glucose, fructose, and sucrose profiles in their extrafloral nectars. On average, glucose made up 56.3 ± 1.8% and 52.7 ± 1.0% of nectar sugars for the third and fifth nodes, respectively, whereas 30.5 ± 1.6% and 31.7 ± 1.2% of the respective nodes' nectar sugars were fructose, and the remaining 13.2 ± 1.5% and 15.7 ± 1.4% of nectar sugars were sucrose. The model for the proportion of glucose was the only model in which node helped to explain enough variance to be of potential importance (Fig. 3.5d). However, even in this case, there was no significant difference between the two nodes when their proportions of glucose were compared using an orthogonal contrast (Table A.2).

3.4 Discussion and conclusions

3.4.1 Differences in extrafloral nectar production between cultivars

The results of the orthogonal contrasts did not support the hypothesis of a consistent difference in total nectar sugar production by EFNs between cultivars with tannins present, compared to those with tannins absent (Fig. 3.1). However, statistical modelling of refractometer measurements did suggest that both cultivar and node significantly affected the mass of nectar sugar produced per stipule, and that the difference between the lower and upper node sampled was in part dependent on the cultivar. The greater average amount of sugar produced by the upper node compared to the lower was likely a result of the plants' growth (BBCH code 11 to 13 or 14) between sampling times (Lancashire *et al.* 1991, Weber and Bleiholder 1990), which were approximately ten days apart. The effect of node may have been especially pronounced since the newly germinated plants grew much more rapidly relative to their overall size than older plants. The node did not appear to have as much of an effect on SSNS-1 as the other three cultivars,

which all showed very similar patterns, and were not statistically different from one another. The apparent reduced effect of node on SSNS-1 was likely due to the slower growth of this cultivar, and smaller EFNs relative to the other cultivars, which occurred in plants grown outdoors as well as those grown in the growth chamber.

Similarly, one *V. faba* cultivar (Tibo) of Kołtoski's (1996b) study did not produce as much extrafloral nectar sugar as the other five cultivars studied. The average sugar mass found per EFN was 45 µg for Tibo, which was much less than the 76 µg, 75 µg, 77 µg, and 80 µg of sugar found in the Dino, Grot, Nadwiślański, and Stego cultivars, respectively (Kołtowski 1996b). Although cultivar Gryf was only sampled for one year, it too had a similar average of 84 µg per EFN. Differences in extrafloral nectar sugar production were not due to differences in tannin production, as cultivars Dino, Nadwiślański, and Tibo have similar tannin content with one another (Skrzypek *et al.* 2012), and are comparable in tannin content to many North American *V. faba* cultivars (Duc *et al.* 1999). The Polish cultivars of *V. faba* produced less extrafloral nectar, on average, than the cultivars used for this study; however, in the Polish study all cultivars were grown outdoors, with breathable and nearly transparent coverings to exclude insects (Kołtowski 1996b). The current study's findings support previous conclusions regarding the importance of relatively high heat and humidity in extrafloral nectar production (Kołtowski 1996b), which could explain differences found between locations, greenhouse and outdoor experiments, and the effect of localized factors such as caged compared to open plots. It is unknown whether or not there are morphological predictors of the total nectar sugar produced by EFNs of different cultivar, whereas floral nectar sugar production in *V. faba* is known to be correlated with the number of modified stomata on the floral nectary projections (Davis and Gunning 1991).

Cultivars were not sampled for extrafloral nectar outside in this study as the nectar proved too viscous to flow up the micropipettes. The plants were instead grown in a growth chamber, which provided constant but altered growing conditions, such as increased humidity, and allowed plants to be grown during the winter. Due to a thrips infestation, seedlings of *V. faba* grown in the growth chamber were also subjected to greater herbivory than plants grown outside, which can result in increased extrafloral nectar production in some plants, but not *V. faba* (Heil 2004, Mondor *et al.* 2013). Despite attempts to employ pest control measures, thrips were found to have selectively consumed extrafloral nectary tissue in each trial, which prevented the EFNs

from secreting nectar once they were visibly damaged. As a result, extrafloral nectar could not be sampled beyond node 5 from the majority of plants within a month after planting the seeds, as EFNs were destroyed too rapidly after being produced. Early termination of the trials also prevented floral nectar from being measured concurrent to extrafloral nectar, as the plants were not in bloom yet.

Total extrafloral nectar sugar measured using refractometer readings (used above) was also compared to total sugar measurements derived from HPLC analyses (Fig. 3.2). The results did not reveal any statistical difference in the average nectar sugar mass per stipule between the two methods (ie., 137.6 ± 10.0 µg for the combined capillary-refractometer method compared to 147.6 ± 15.5 µg for the wicked nectar drops analysed by HPLC), supporting future comparisons using data obtained from either method. The small difference between the two averages for nectar sugar mass may have been due to the inclusion of the occasional occurrence of nectarless samples (3/128) within the refractometer-derived data not encountered with acquisition of the HPLC data, as well as natural variation within the population being sub-sampled.

As with total production of nectar sugars, the presence of tannins did not appear to be a significant factor in nectar sugar concentration (Fig. 3.4). Instead, cultivars Fatima, Snowbird, and Snowdrop all had similar and significant increases in nectar sugar concentration from node three to node five, whereas SSNS-1 did not have a significant increase from one sampled node to the next. The average NCW ranged from 30 - 37 % for all cultivars, and was higher than the 25% sugar concentration reported for the Outlook cultivar by Davis *et al.* (1988), which was also measured in a greenhouse. Measurements taken outdoors were reported to be 40-60% for the six Polish cultivars mentioned above (Kołtowski 1996b), and for four previously popular Canadian cultivars (Ackerperle, Diana, Erforida, and Herz Freya), the average sugar concentration was 16 – 25% for open plots, and 25 – 39% for caged plots (Malaipan 1979). Nectar concentrations measured in the current study therefore fit well within the findings of previous studies.

Unlike nectar sugar concentration and mass, nectar volumes were more variable between cultivars, although many cultivars were still statistically similar to one another. SSNS-1 had the lowest nectar volumes at both nodes sampled, whereas Fatima was the only cultivar to have a statistical difference in average nectar volume between the nodes sampled (Fig. 3.3). However, the average nectar volume measured for Fatima was not statistically different from samples of both two tannin-free cultivars, suggesting that differences in tannin content do not influence

nectar volume either. The average standing nectar volumes for all four cultivars in this study were similar to the nectar volumes found in the four different cultivars examined by Malaipan (1979), which ranged from an average of 0.16 - 0.45 µL per stipule. In Malaipan's study, only one cultivar (Herz Freya) had a statistically greater extrafloral nectar volume than the other three cultivars, which were otherwise not statistically different.

3.4.2 Variations in extrafloral nectar composition

When contrasts between cultivars were performed for each sugar's proportion, there did not appear to be differences in sugar proportions based on the cultivar's tannin content (Fig. 3.5a-c), and there were few statistical differences between individual cultivars. For the proportion of sucrose, the Snowdrop cultivar had the smallest proportion (Fig. 3.5a), Snowbird the largest, and both Fatima and SSNS-1 fell between them, resulting in the average proportion of sucrose being very similar between cultivars with and without tannins (14% and 15% sucrose, respectively). Between different cultivars, the average proportions of an individual sugar were similar, as they were within 10% of one another. Overall, the proportions of individual sugars within the extrafloral nectar varied much less between cultivars than nectar sugar concentration and volume did, although there were relatively large variations in nectar sugar proportions between individuals within the various cultivars.

In addition to differences in sugar proportions between cultivars, the node sampled was also left in the general linear model for the proportion of glucose. An orthogonal contrast between the two nodes failed to find any difference between them, and the average proportion of extrafloral nectar sugar that was glucose differed by less than 4% (Fig. 3.5d). The statistical difference between nodes found during model selection was smaller in effect size than even the differences between cultivars, and would likely be eliminated with a slightly larger sample size. Therefore, even for the proportion of glucose, node is unlikely to represent a difference of biological significance.

Overall, more than half of *V. faba* extrafloral nectar sugars, by weight, were found to be glucose, with the remainder of nectar sugars being composed of twice as much fructose as sucrose (Fig. 3.5a-c). This places the study in closest agreement with previous thin-layer chromatography work, which had found *V. faba* extrafloral nectar was composed of mostly glucose and fructose (Davis *et al.* 1988). In contrast to overall nectar sugar concentrations,

however, information on nectar sugar composition was previously conflicting, and much less detailed. Previous use of HPLC measurements had provided the most details, including the mM of each sugar per 10 mg dry weight of stipular tissue (Irvin *et al.* 2007), which as a percentage of total sugars by weight was 40.4% glucose, 5.4% fructose, 46.1% sucrose, and 8.1% inositol (a polyol). However, Irvin *et al.*'s measurements were used to approximate extrafloral nectar through the sampling of stipular tissue, and therefore may have instead provided insight into how extrafloral nectar composition is altered post-secretion in *V. faba*. Aside from the greater, but still low polyol content, the above nectar composition could have ended up being similar to the current study through invertase activity, which can alter nectar composition after secretion through the breakdown of sucrose into glucose and fructose (Heil *et al.* 2005b).

Invertase activity does not explain the uneven ratio of glucose to fructose however, which was 3.8 µg glucose : 2.1 µg fructose : 1.0 µg sucrose in the current study. Microbial activity is known to alter glucose:fructose ratios post-secretion (Lüttge 1961, De Vega and Herrera 2013), but the ratio of glucose:fructose heavily favoured glucose in extrafloral nectary tissues as well (Irvin *et al.* 2007), suggesting that the glucose:fructose ratio may not be 1:1 prior to secretion. Oligosaccharides have also been suggested to cause uneven glucose:fructose ratios (Lüttge 1962), however none, other than sucrose, appear to have been found in *V. faba* extrafloral nectar. The uneven ratio is therefore more likely to have resulted from intracellular processes, such as the sugars cycling through part or all of the glycolysis, gluconeogenesis, and pentose phosphate pathways (Wenzler *et al.* 2008).

Nectar sugar proportions can also reveal information regarding the plant's visitors, and although far more literature examines the carbohydrate composition of floral nectar, extrafloral nectar sugars are no exception to this. Potential invertase production by *V. faba* may split the majority of the secreted sucrose molecules into their constituent sugars, which are more easily digested by insects. Some ants produce relatively little invertase themselves (Ayre 1967), and parasitoid wasps may not necessarily be able to digest sucrose at all (Wanner *et al.* 2006). Therefore, a relatively low sucrose content allows the nectar to be consumed by a wider variety of predatory and parasitoid insects which lack any specialized relationships with the plant. Although some ants do prefer sucrose-rich extrafloral nectar, not all ants have a preference for any particular nectar sugars (Blüthgen and Fiedler 2004, Cornelius *et al.* 1996). Low sucrose proportions in *V. faba*'s extrafloral nectar suggests that the plant likely lacks specialized

relationships with specific EFN visiting insects, and instead forms more generalized mutualistic associations with wasps and ants.

4 General discussion and conclusions

Vicia faba, like many other legumes identified in Chapter 1, attracts a variety of insects to its extrafloral nectaries. Many of these insects would otherwise be unlikely to visit the plant, as the predators and parasitoids attracted to the extrafloral nectar are not able to access the plants' floral nectar. One of the goals of this study was to examine any differences in insect identity and abundance that visited EFNs of *V. faba* cultivars with or without tannins, which when present make the extrafloral nectaries far more visible (Fig. 1.1a,b). Overall, there were no consistent differences in insect assemblages or their frequency of visits to cultivars with tannins, compared to those without. Similar results came about from studying the mass, concentration, and composition of extrafloral nectar sugars in the four different cultivars.

Instead, minor differences appeared between the cultivars, which would be a useful level at which to study *V. faba*'s insect visitors in the future. Geographical and climatic differences are likely responsible for most of the variations between different studies of the abundance and species of insects found to visit *V. faba*'s floral nectaries, although there was significant overlap at both the generic and species level of the visitors between studies. Interestingly, bees of the family Apidae were the main visitors to *V. faba*'s flowers in most studies, even if the ever-present *A. mellifera* are not included (Aouar-sadli *et al.* 2008, Benachour *et al.* 2007, Bond and Kirby 1999, Kendall and Smith 1975, Kołtowski 1996c, Pierre *et al.* 1996). Further work would be needed to determine why other similarly sized bees, such as megachilids, do not seem to visit *V. faba* flowers, despite being the main pollinators used in seed production for other legumes, such as alfalfa. Also of interest was the very low number of parasitoid wasp visitors in this study (even for the nearly ant-free locations) and others (Hetschko 1908, Nuessly *et al.* 2004, Malaipan 1979), compared to the numerous species found by Bugg *et al.* (1989). Although the differences in parasitoid numbers could have resulted from differences in the surrounding vegetation or climate, future research is needed to determine what factors could influence parasitoid-wasp visitor numbers to such a degree, because the wasps can provide a significant reduction in the herbivores present on *V. faba* (Cuautle and Rico-Gray 2003).

Ants were another group of insects which were quite variable in their presence at the EFNs of *V. faba*, which was especially noticeable between this study's two field sites. The timing and frequency of tilling were the main differences between the ant-filled and ant-free plots used during this study, which agrees with previous findings suggesting that farming

practices involving tilling have significant implications on the insect community composition within fields, especially for ground nesting insects (Bugg and Ellis 1990). The vast majority of native bees and ants nest in the soil, which may result in greater populations of beneficial predators and pollinators if the area is left untilled (House and Alzugaray 1989). The effect of tilling on insect communities within cultivated areas would be a very interesting topic to explore further, as the majority of past research has focused on the pest insects reduced by tilling, rather than the effect on the insect community as a whole.

In contrast to the surveys conducted of visitors to EFNs of *V. faba*, insect visitors other than ants were plentiful for the plants grown in a greenhouse. Unfortunately, those visits were conducted entirely by thrips, which appeared to feed exclusively on EFN tissue as long as undamaged EFNs were still plentiful. Targeted feeding on *V. faba* in other studies has also been recently reported, despite the chemical and mechanical defences of the EFNs (Gish *et al.* 2015, 2016). Thrips infestations posed a substantial problem for studying extrafloral nectar, and resulted in the nectar sampling time for this study being significantly shortened for each set of plants, as damage to the EFNs of *V. faba* increased exponentially over time. It is not recommended that future researchers attempt to examine *V. faba* extrafloral nectar unless access to a pest-free and tightly sealed greenhouse unit is available. Due to the minute size of thrips, nearly any openings allow them to infiltrate otherwise isolated growth chambers, especially if adjacent areas are infested with them.

Despite the thrips damage to EFNs of plants inside the greenhouse, thrips did not significantly damage the exposed EFNs of *V. faba* grown outside. However, plants grown outdoors had extrafloral nectar which was too viscous to allow for measurements of volume, as capillary action did not provide enough force for nectar to flow up the micropipettes generally used to measure small volumes of nectar. Wicking of the nectar with filter paper is recommended in this situation (Corbet 2003), though it does not allow nectar volume or concentration to be measured. It is likely that the extrafloral nectar sugar mass model would be more informative if extrafloral nectar sampling could extend until the end of the flowering period, as further sampling throughout the life of *V. faba* would likely eliminate the model's interaction between cultivar and node. Although alternate methods have attempted to infer extrafloral nectar composition from EFN tissue (Irvin *et al.* 2007), EFN tissue and secreted nectar are not

equivalent to one another, as nectar can be significantly altered post-secretion (De Vega and Herrera 2013, Heil *et al.* 2005b, Lüttge 1961).

Variations in the nectar secretion of individual stipules may also prove interesting to examine, though combining extrafloral nectar from both stipules of a node may be a necessity for nectar destined for analysis by HPLC, as EFN is produced in relatively low volumes. The composition of extrafloral nectar sugar from all cultivars examined in this study was over 50% glucose by weight, with fructose comprising the majority of the remaining sugars. This left only a small amount of sucrose, which was not unexpected, given that past research has indicated monosaccharides may be more digestible to some ants and parasitoid wasps (Ayre 1967, Wäckers 2001, Wanner *et al.* 2006), and nectar high in glucose and fructose may help to retain water in the exposed nectar droplet (Corbet *et al.* 1979). It would be interesting to measure the amount of invertase produced by the various ants and wasps which frequent *V. faba*, especially compared to the pollinators visiting the more sucrose-rich floral nectar, which contained a mass of sucrose over four times greater than that of hexose sugars for *V. faba* cv. Aquadulce (Davis and Gunning 1991). The uneven ratio of glucose and fructose was more unexpected, the cause of which would require further research. Few other ratios of extrafloral nectar sugars are available, so additional study would enable broader comparisons of extrafloral nectar sugars with the sugars in floral nectar.

Perhaps the most surprising aspect of the study was the consistency of the ants' choice of EFNs to visit along the stem, with a region containing the Primary Active EFNs only a few centimetres in length accounting for the vast majority of the visits, despite the plants growing well over 1 m tall. This localization of feeding activity by ants to EFNs of *V. faba* occurred regardless of ant species, plant cultivar (tannin-free or -rich), or plant age. It is unknown whether this region of enhanced extrafloral nectar production is a by-product of nearby vegetative or fruit growth, or specifically secreted to draw ants and wasps to that particular part of the plant. Further study into the physiological reasons behind this extrafloral nectar secretion phenomenon could yield informative results, especially concerning the importance of drawing predatory and parasitoid insects to that relative location. In addition to visiting stipular EFNs, ants were also observed feeding on tendril EFNs attached to the leaves stemming from the Primary Active EFNs, which may warrant further research, as past studies have tended to focus exclusively on the stipular EFNs of *V. faba*.

However, regarding the frequency of visits by various taxa, once again, the presence of tannins did not appear to have any effect. This finding seems to indicate that the visibility of the EFNs had little to no effect, as EFNs near the top of tannin-containing plants were easily visible from a couple metres away, whereas tannin-free cultivars had EFNs which were difficult to locate even up close, suggesting insects such as ants may have relied entirely on chemosensory or other non-visual indicators to detect the nectar. Further research would be needed to provide a convincing purpose for the tannins in the EFNs, such as a reduction in the number of small insects selectively consuming, and thereby destroying, the EFN tissue, as tannins are known to reduce herbivory in other plants (War *et al*. 2012). Overall, the presence or absence of tannins did not appear to effect the extrafloral nectar, or insect visitors to the extrafloral and floral nectaries, in any way. The increased digestibility of tannin-free *V. faba* to farmed animals such as chickens, therefore, does not appear to come at a price, at least in terms of the beneficial insects attracted to the plants.

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Appendix

Table A.1 Comparative values for statistical models of different ant species' presence/absence at patches of *Vicia faba* (n = 144); bee species' presence/absence at patches of flowering *Vicia faba* (n = 64); extrafloral nectar volume, concentration, and sugar mass measured through refractometry (n = 128); and high performance liquid chromatography (HPLC) measurements of extrafloral sugar mass and composition (n = 50). All models of insect species' presence/absence are generalized mixed models, which always include random factors, whereas the models of extrafloral nectar characteristics are general linear models, which lack random factors. Model types in the table include: Null models that lack explanatory variables; Explanatory models that include cultivar as an explanatory variable; Maximum models that include all explanatory variables of interest; and Min. (minimum) Adequate models that include only those explanatory variables which produce the model of best fit. Probability values (*p*) produced from Chi-squared analyses are provided for selected models, which were each compared with the model directly above them in the table. Degrees of freedom (DF), residual deviance (Deviance), and Akaike's Information Criterion (AIC) values are also provided for each model.

Data	Type of Model	DF	AIC	Deviance	*p*
Formica lasioides	Null	139	41.3	31.3	
	Explanatory	136	40.6	24.6	0.083
F. neoclara	Null	139	171.9	161.9	
	Explanatory	136	175.4	159.4	0.474
F. podzolica	Null	139	139.9	129.9	
	Explanatory	136	137.2	121.2	0.034
Lasius pallitarsis	Null	139	110.3	100.3	
	Explanatory	136	101.2	85.2	0.002
Myrmica brevispinosa	Null	139	51.1	41.1	
	Explanatory	136	34.6	18.6	<0.001
Apis mellifera	Null	59	65.8	55.8	
	Explanatory	56	66.8	50.8	0.170
Bombus nevadensis	Null	59	51.6	41.6	
	Explanatory	56	57.2	41.2	0.937
Sugar mass:	Null	127	1577	1628930	
Refractometery	Min. Adequate	120	1527	988414	<0.001
	Maximum	112	1534	922426	0.439
Nectar Volume	Null	127	0.04	7.3	
	Min. Adequate	120	-26.5	5.3	<0.001
	Maximum	112	-22.7	4.8	0.206
Nectar Concentration	Null	127	1052	26983	
	Min. Adequate	119	1014	17643	<0.001
	Maximum	112	1020	16607	0.436
Sugar mass: HPLC	Null	49	615.6	600760	
	Min. Adequate	46	601.7	403648	<0.001
	Maximum	34	606.6	275316	0.252

Table A.1 (cont.)

Data	Type of Model	DF	AIC	Deviance	*p*
% Glucose	Null	49	343.7	2611	
	Min. Adequate	45	334.1	1837	0.003
	Maximum	34	350.2	1633	0.953
% Fructose	Null	49	339.5	2403	
	Min. Adequate	46	335.6	1971	0.027
	Maximum	34	349.0	1593	0.765
% Sucrose	Null	49	345.3	2698	
	Min. Adequate	46	331.2	1805	<0.001
	Maximum	34	340.2	1336	0.473

Table A.2 Resultant *p*-values, after Holm-Bonferroni sequential corrections, from ranked orthogonal contrasts of different cultivar and node combinations from generalized mixed models of ant visits to *Vicia faba* extrafloral nectaries (n = 144), as well as general linear models of extrafloral nectar volume, concentration, and sugar mass derived from microcapillary sampling and refractometry measurements (n = 128), and high performance liquid chromatography (HPLC) measurements of extrafloral sugar mass and composition (n = 50).

Data	Contrast	*p*
Formica podzolica	Fatima + Snowbird	0.291
	Fatima + Snowdrop	0.037
	Snowbird + Snowdrop	0.231
Lasius pallitarsis	Snowbird + SSNS-1	0.532
	Fatima + Snowbird	0.081
	Fatima + SSNS-1	0.109
	Snowbird + Snowdrop	0.048
	Snowdrop + SSNS-1	0.065
Sugar mass: Refractometery	SSNS-1_3 + SSNS-1_5	0.225
(Fig. 3.1)	Snowbird_3 + SSNS-1_3	0.024
	Snowbird_3 + Snowdrop_3	0.216
	Snowdrop_5 + SSNS-1_5	0.006
	Snowbird_3 + Snowdrop_5	0.264
	Snowbird_3 + Snowbird_5	0.023
	Fatima_5 + Snowdrop_5	0.428
Sugar mass: Refractometery	Fatima + SSNS-1	0.299
(Fig. 3.2)	Snowbird + SSNS-1	0.012
	Snowbird + Snowdrop	0.108
Nectar Volume	Snowbird_5 + SSNS-1_3	0.135
	Snowbird_3 + SSNS-1_5	0.013
	Fatima_3 + Snowbird_3	0.080
	Snowbird_3 + Snowdrop_3	0.117
	Fatima_5 + Snowdrop_5	0.109
	Fatima_5 + Snowdrop_3	0.035

Table A.2 (cont.)

Data	Contrast	*p*
Nectar Concentration	Snowdrop_3 + SSNS-1_5	0.058
	Fatima_3 + Fatima_5	0.019
	Fatima_5 + Snowbird_3	0.058
	Snowbird_3 + Snowdrop_5	0.012
	Snowdrop_5 + SSNS-1_3	0.072
	Snowbird_5 + SSNS-1_5	0.004
	Fatima_5 + Snowbird_5	0.092
Sugar mass: HPLC	Snowdrop + SSNS-1	0.340
	Snowbird + Snowdrop	0.007
% Glucose (Fig. 3.5c)	Fatima + Snowbird	1.000
	Snowbird + SSNS-1	0.035
	Fatima + SSNS-1	0.075
	Snowdrop + SSNS-1	0.768
	Fatima + Snowdrop	0.054
% Glucose (Fig. 3.5d)	Node 3 + Node 5	0.635
% Fructose	Snowdrop + SSNS-1	0.121
	Fatima + SSNS-1	0.036
	Fatima + Snowbird	0.064
	Fatima + Snowdrop	0.483
% Sucrose	Fatima + Snowdrop	0.136
	Snowdrop + SSNS-1	0.066
	Fatima + SSNS-1	0.576
	Snowbird + SSNS-1	0.002

Table A.3 Statistical models examined to determine the minimum adequate model for *Vicia faba* extrafloral nectar sugar mass, assessed by nectar refractometry. Values for Akaike's Information Criterion (AIC) and degrees of freedom (DF) were used to evaluate the models' suitability.

General Linear Model	AIC	DF
Null	1577	127
Cultivar*Trial*Node	1534	112
Cultivar*Trial+Node	1531	119
Cultivar+Trial*Node	1530	121
Cultivar*Node+Trial	1529	119
Cultivar*Node	1527	120

Table A.4 Statistical models examined to determine the minimum adequate model for *Vicia faba* extrafloral nectar sugar mass, measured by high performance liquid chromatography (HPLC). Values for Akaike's Information Criterion (AIC) and degrees of freedom (DF) were used to evaluate the models' suitability.

General Linear Model	AIC	DF
Null	615.6	49
Cultivar*Trial*Node	606.6	34
Cultivar*Trial+Node	600.7	41
Cultivar+Trial*Node	603.1	43
Cultivar*Node+Trial	606.1	41
Cultivar+Trial+Node	602.6	44
Cultivar+Node	600.8	45
Cultivar	601.7	46

Table A.5 Statistical models examined to determine the minimum adequate model for *Vicia faba* extrafloral nectar volume per stipule, assessed by microcapillary sampling. Values for Akaike's Information Criterion (AIC) and degrees of freedom (DF) were used to evaluate the models' suitability

General Linear Model	AIC	DF
Null	0.04	127
Cultivar*Trial*Node	-22.7	112
Cultivar*Trial+Node	-22.8	119
Cultivar+Trial*Node	-28.0	121
Cultivar*Node+Trial	-27.4	119
Cultivar*Node	-26.5	120

Table A.6 Statistical models examined to determine the minimum adequate model for *Vicia faba* extrafloral nectar concentration by weight per stipule, assessed by nectar refractometry. Values for Akaike's Information Criterion (AIC) and degrees of freedom (DF) were used to evaluate the models' suitability.

General Linear Model	AIC	DF
Null	1052	127
Cultivar*Trial*Node	1020	112
Cultivar*Trial+Node	1023	119
Cultivar+Trial*Node	1023	121
Cultivar*Node+Trial	1014	119

Table A.7 Statistical models examined to determine the minimum adequate model for the proportion of glucose in *Vicia faba* extrafloral nectar sugars, measured by high performance liquid chromatography (HPLC). Values for Akaike's Information Criterion (AIC) and degrees of freedom (DF) were used to evaluate the models' suitability.

General Linear Model	AIC	DF
Null	343.7	49
Cultivar*Trial*Node	350.2	34
Cultivar*Trial+Node	340.8	41
Cultivar+Trial*Node	337.5	43
Cultivar*Node+Trial	340.8	41
Cultivar+Trial+Node	336.1	44
Cultivar+Node	334.1	45

Table A.8 Statistical models examined to determine the minimum adequate model for the proportion of sucrose in *Vicia faba* extrafloral nectar sugars, measured by high performance liquid chromatography (HPLC). Values for Akaike's Information Criterion (AIC) and degrees of freedom (DF) were used to evaluate the models' suitability.

General Linear Model	AIC	DF
Null	345.3	49
Cultivar*Trial*Node	340.2	34
Cultivar*Trial+Node	334.8	41
Cultivar+Trial*Node	331.8	43
Cultivar*Node+Trial	335.3	41
Cultivar+Trial+Node	330.5	44
Cultivar+Node	330.5	45
Cultivar	331.2	46

Table A.9 Statistical models examined to determine the minimum adequate model for the proportion of fructose in *Vicia faba* extrafloral nectar sugars, measured by high performance liquid chromatography (HPLC). Values for Akaike's Information Criterion (AIC) and degrees of freedom (DF) were used to evaluate the models' suitability.

General Linear Model	AIC	DF
Null	339.5	49
Cultivar*Trial*Node	349.0	34
Cultivar*Trial+Node	338.0	41
Cultivar+Trial*Node	339.4	43
Cultivar*Node+Trial	341.9	41
Cultivar+Trial+Node	337.4	44
Cultivar+Trial	336.0	45
Cultivar	335.6	46